FREDERICK GOWLAND HOPKINS

(June 20, 1861 - May 16, 1947)

During 1950 THE JOURNAL OF NUTRITION will carry on its front cover a picture of Frederick Gowland Hopkins. The photograph [1] used for this also appears as a frontispiece in this first issue of volume 40. In this manner THE JOURNAL OF NUTRITION pays tribute to another distinguished scientist who did much to lay the foundations of the modern science of nutrition.

Frederick Gowland Hopkins was born in Eastbourne, England, June 20, 1861; he died in Cambridge May 16, 1947, about a month short of his 86th birthday. His father died when he was a small child. He was privately educated. At the age of 17 years he took his first paid position, that of clerk in an insurance office, but held it for only 6 months. This was followed by three years of work as an assistant in a commercial laboratory devoted to analytical practice. As he wrote in his autobiography,[2] "These years taught me something perhaps which might not have been learnt elsewhere, for instance, how to obtain results in the shortest possible time; but intellectually they were nearly sterile years." Aware of the need for a proper scientific education the young man then went to the Royal School of Mines hoping to be a pupil of the elder Frankland, who, however, did little teaching that term. Actually Hopkins heard only two — or at most three — lectures by Frankland, and makes the interesting comment: "yet those lectures were the only educational lectures on chemistry to

[1] Used by kind permission of J. Russell and Sons, 63 Baker Street, London.

[2] This autobiography appears as the opening chapter of the Commemoration Volume entitled *Hopkins and Biochemistry 1861–1947*, published by W. Heffer and Sons, Limited, Cambridge. An excellent and informative obituary was also published in *Lancet*, May 24, 1947, pp. 728–731.

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which I have ever listened!" At the end of the term Hopkins was invited to become an assistant to the young Dr. Percy Faraday Frankland in a private laboratory just being started. This was accepted, but the work lasted for only 6 months. He then attended lectures at University College, London, and successfully passed an examination required in order to qualify for membership in the Institute of Chemistry. This led to an invitation from Dr. (afterwards Sir Thomas) Stevenson, Medical Jurist at Guy's Hospital and Home Office Expert, to become an assistant in his laboratory. The experiences in this laboratory finally led Hopkins to seek further education, more particularly to qualify for medical work. This was accomplished by study at London University while earning his living as a chemist at Guy's Hospital. In 1894 he "obtained the Conjoint Board Qualification and the London M.B." This led to an appointment to the school staff where he acted as one of the demonstrators in practical physiology in the early days of Starling's appointment as lecturer. What is particularly interesting to note is that during this time he did his best to "keep some sort of research going."

In 1898, the great Sir Michael Foster, Professor of Physiology at Cambridge, invited Hopkins to come to Cambridge and develop teaching and research in what was then called chemical physiology. He hesitated to accept such an appointment, however, because, as he himself remarked, he was "without training as a specialized biochemist. I had never paid the then orthodox visit to a German laboratory and, indeed, had had no contact with any master of the subject." Encouraged by his wife whom he had married only a short time before, he accepted the invitation and went to Cambridge where he spent the rest of his scientific career.

The first publication by Hopkins consisted of a note contributed to a naturalists' magazine *The Entomologist* in 1878 when he was 17 years old. It dealt with some observations made of the little bombardier beetle *Brachinus crepitans*. Eleven years later, while being articled for service in the analysts' laboratory, he displayed further interest in entomo-

logical problems in the form of a preliminary report on chemical studies of pigments present in the wings of the common English brimstone butterfly. In a later paper published in the *Philosophical Transactions* of the Royal Society, full details are given, and it appears that he isolated a pigment and proved it to be a uric acid derivative. This work also led to an improved method for estimating uric acid. At that time the uric acid output was considered to be influenced by dietary protein. This work therefore led Hopkins to perform experiments with purified proteins and artificial diets. The first great discovery made in this connection was the isolation and identification of tryptophan with S. W. Cole in 1901. This was followed later by the earliest researches on nutritionally essential amino acids, carried out with Edith G. Willcock, experiments showing that zein could be improved nutritionally by supplementation with tryptophan. This line of investigation led Hopkins to express the view that in addition to the well-known nutrients proteins, carbohydrates, fats and minerals, there are *unidentified accessory food factors*. He went even further, and suggested that these same factors are the ones needed to prevent the development of deficiency diseases.[3] It was this work which led many years later, in 1929, to the Nobel Prize in Medicine being bestowed on Hopkins jointly with Eijkman.

Hopkins made many other notable contributions to the science of biochemistry and nutrition. In 1907 he collaborated with Fletcher in studies of muscle physiology, serving as the chemist member of the team. They examined the lactic acid produced by frog muscle under various conditions. They showed that lactic acid is produced during muscular contraction, and that during rest with ample supply of oxygen the lactic acid disappears; on the other hand, under anerobic conditions the acid accumulates in the muscle.

The work leading to the discovery and isolation of glutathione constitutes another interesting chapter in the story of Hopkins' researches. In his first paper on this topic, pub-

[3] See the Lecture in 1906 to the Society of Public Analysts.

lished in 1921, Hopkins wrote: "My attention was directed to the subject in two separate ways. Some years ago I was endeavoring to discover if vitamins were to be found among sulphur-containing compounds, and was led part of the way toward the separation of the substance now described. A little later, acting on the suggestion that acid formation in muscle is a necessary factor in contraction, I wished to discover if by chance, in the absence of carbohydrate, acetoacetic acid from fat might function instead of lactic acid. This led me to apply the nitroprusside test to tissues. At this time Arnold's papers had not yet appeared and I was ignorant of Heffter's publications. The above-mentioned inquiries were nugatory but they led to the present one." The idea that some vitamins might perhaps be found among sulphur-containing compounds, although not substantiated by the sulfhydryl line of research, was shown to be correct not many years later through the work of Williams and others who isolated and synthesized thiamine. The Hopkins investigation finally resulted in the isolation of glutathione and the submission of evidence believed to indicate that it was a dipeptide, a conclusion later shown to be incorrect by Hunter and Eagle in America who believed it to be a tripeptide. This led Hopkins to improve his method of isolation and to perform various additional experiments the results of which demonstrated the tripeptide nature of the compound. Studies of the properties of glutathione naturally led Hopkins into the field of tissue oxidations and reductions, and the publication of many papers in this area of interest. The bibliographic list of all of his publications (as given in the 1948 Commemoration Volume), which includes of course many lectures and similar papers, totals 132 items.

Hopkins received many distinguished honors. As a comparatively young man, in 1905, he was elected a Fellow of the Royal Society; in 1931 he served as its president. In 1914 a special professorship in biochemistry was established for him at Cambridge University. Seven years later he was appointed the first holder of the Sir William Dunn Chair of Biochemistry at Cambridge and head of the associated Sir William Dunn

Institute. He was knighted in 1925. In 1929 came the very high honor of sharing the Nobel Prize in Medicine with Eijkman. At the Harvard Tercentenary celebration in 1936 Hopkins was one of the 66 world's leading scientists and scholars to receive a Harvard honorary degree. In addition, numerous medals and similar honors came to him, such as, to mention but a few, the Royal medal and the Copley medal from the Royal Society, Baly medal of the Royal College of Physicians, Society of Apothecaries medal, and the Order of Merit (1935).

Sir Frederick Hopkins' home life was a happy one. In 1898 he was married to Jessie A. Stevens. This union was blessed by three children, a son Frederick who became a doctor, a daughter Barbara Holmes who has achieved independent recognition as a research biochemist, and another daughter Jaquetta Hawkes who has made archeology her special field of interest. As a laboratory mentor Sir Frederick was kind and gentle in dealing with students and associates. He found it almost impossible to be ruthless, and seemed somehow always to be able to find something appreciative and worthwhile to say about the work of the other party. This spirit of helpfulness was much appreciated by the many students and visiting scholars who came to the Cambridge Laboratory from foreign countries. Their memories of the man occupied a preeminent place in their memories of the Laboratory where they had worked, and no doubt contributed in considerable measure to insuring the success of the first International Congress in Biochemistry held in Cambridge in 1949, two years after his passing. Publication of the Commemoration Volume entitled *Hopkins and Biochemistry 1861–1947* was made part of the program of the Congress, so to speak. Students interested in biographies of biochemists and nutritionists will find this volume a mine of valuable information concerning one of the great men and laboratories in their field.

G. R. C.

PRELIMINARY OBSERVATIONS UPON FACTORS INFLUENCING CELLULOSE DIGESTION BY RUMEN MICROORGANISMS [1]

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The physiology of digestion of ruminants is unique among domestic animals in that bacterial enzymes play an integral part. Actual digestion of cellulose and related compounds found in hays and roughages consumed by cattle and sheep is dependent upon enzymes elaborated by microorganisms living within the digestive tract.

During the past several years digestion experiments have been under way at this laboratory in an attempt to determine fundamental concepts regarding rumen microorganisms and the digestion of roughages fed to cattle. In these studies (Burroughs and Gerlaugh, '49; Burroughs et al., '49a) protein was found to be a beneficial factor in roughage digestion, especially where starch or a starchy grain was included in the ration. The addition of cornstarch to certain roughages (Burroughs et al., '49b) had little or no influence upon the digestion of good quality alfalfa hays, whereas a substantial lowering in digestion was noted when starch was fed with poor quality roughage. Further studies (unpublished data) indicated that a water extract from dehydrated alfalfa meal

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or the ash from alfalfa meal enhanced the dry matter digestibility of a poor quality roughage when fed in a ration containing corn starch.

To obtain more detailed information on the factors involved in roughage digestion by rumen microorganisms, it seemed plausible to devise an artificial rumen somewhat similar to that described by Marston ('48) which could be used in laboratory studies. The purpose of this paper is to describe the method employed in attempting to simulate rumen conditions in the laboratory and to note the influences of various materials upon cellulose digestion in preliminary use of the method.

EXPERIMENTAL

Continuous 36-hour fermentation periods were used in which the original starting inoculum was a mixed culture of organisms taken directly from the rumen of cattle. The inoculum employed in the second and succeeding 36-hour periods consisted of one-half the residue from the preceding fermentation. Cellulose digestion was determined chemically by the method of Crampton and Maynard ('38) on the half portions not used as inoculum, and Gram stains of the microflora were observed at the beginning and end of each fermentation period.

A battery of 6 glass fermentation flasks of 500 ml capacity were placed in a round constant temperature water bath maintained at 40°C. Each flask was fitted with a no. 10 rubber stopper containing three holes. Two of the holes in each stopper served as inlet and outlet, respectively, for a continuous flow of carbon dioxide gas, while the third hole was fitted with a small removable stopper for pH regulation. The flasks were connected with tubing such that one source of carbon dioxide served all flasks. The flow of carbon dioxide through the flasks was at least 120 bubbles per minute.

The materials initially placed in each fermentation flask included 1 gm of filter paper, 50 ml of salt solution, 140 ml of rumen ingesta liquid, and distilled water to make a total

volume of 350 ml. In certain flasks a portion of the distilled water was replaced with an equal volume of the material being studied.

Three materials, namely, alfalfa juice ash, autoclaved rumen liquid, and autoclaved water extract of manure, were studied. The alfalfa juice ash was prepared by extracting dehydrated alfalfa meal with warm water three times, evaporating the liquid to dryness, and ashing at dull red heat in a muffle furnace.

The autoclaved rumen liquid was prepared by first obtaining the raw material from the steer, in a manner similar to the way in which the original inoculum was collected, as described below. Enough hydrochloric acid was added to reduce the pH to 5.0. The acidified liquid was autoclaved at 15 lb. for 60 min. in an unstoppered bottle. After cooling it was centrifuged 15 min. at 3,000 R.P.M. in an angle centrifuge from which the liquid was saved and the heavy material discarded. The liquid was then neutralized with 0.5 M sodium carbonate and stored in the refrigerator.

The autoclaved manure extract was prepared by collecting freshly defecated manure from the animal from which the original rumen inoculum was obtained. Five pounds of manure were mixed thoroughly with 7½ lb. of warm tap water. The watered manure was transferred to a heavy cotton bag and the liquid portion squeezed out with the aid of a lard press. This liquid was acidified, autoclaved, centrifuged, and neutralized in a manner similar to the autoclaved rumen liquid before being stored in the refrigerator.

The filter paper, Whatman's analytical, was ground in a Wiley mill through the medium screen. The salt solution was varied in different flasks but was characterized as either a complex or a simple solution. The simple solution contained, as the only source of nitrogen, either ammonium sulfate or urea. The complex solution was patterned after the composition of sheep saliva (McDougall, '49), with the addition

of trace elements known to be essential in animal nutrition. It had the following composition:

Sodium phosphate, monobastic, gm	52.50
Sodium bicarbonate, gm	52.50
Ammonium sulfate,[2] gm	37.50
Potassium chloride, gm	7.50
Sodium chloride, gm	7.50
Magnesium sulfate, gm	2.25
Calcium chloride, gm	0.75
Ferrous sulfate, gm	0.15
Manganous sulfate, gm	0.08
Zinc sulfate, gm	0.08
Copper sulfate, gm	0.04
Cobalt chloride, gm	0.02
Distilled water, ml	2,000

The rumen ingesta liquid was obtained by means of a large bore stomach tube inserted into the reticulum area of a steer fed an alfalfa hay ration. After fasting the animal 16 hours approximately 1 l of ingesta was removed, immediately squeezed through 4 thicknesses of a no. 50 grade cheesecloth, and the resulting liquid placed in the fermentation flasks as rapidly as possible. Each flask was previously charged with all ingredients except the inoculum and the flow of carbon dioxide started 30 min. before the ingesta juice was added, thus assuring proper initial temperature and anaerobic conditions.

Immediately after the inoculum was added, carbon dioxide was allowed to flow through the flasks for 15 min. The flow was then stopped for a few minutes and samples were drawn for Gram stains and pH measurements. Regulation of the hydrogen ion concentration was obtained with additions of 0.5 M sodium carbonate as the fermentation proceeded, maintaining it in the range between 6.2 and 6.8.

The materials added to each flask at the beginning of the second and succeeding fermentations included the inoculum, 1 gm of filter paper, 25 ml of salt solution and sufficient distilled water to make a total volume of 350 ml.

[2] In certain solutions 17.04 gm of urea were substituted for the ammonium sulfate.

RESULTS

Experiment 1

The cellulose digested in flasks 1 through 6 during the first 7 36-hour periods is presented in table 1. Flask 6 contained ammonium sulfate but no other added minerals. Cellulose digestion in this flask (68%) during the first 36-hour period was very satisfactory but not quite as high as that occurring in the remaining flasks. During the second 36-hour period, cellulose digestion in flask 6 (9%) dropped severely, which was followed by a further drop in the third fermentation. No cellulose digestion occurred in this flask from the 4th through the remaining 36-hour periods.

The materials placed in flask 5 were similar to those in flask 6 except that the former had 0.5 gm of alfalfa juice ash added at the beginning of each 36-hour period.

Cellulose digestion was increased somewhat as a result of the alfalfa ash additions, as can be seen by comparing the first two 36-hour periods (table 1) of flask 5 with the corresponding periods for flask 6. Beyond the third period, cellulose digestion also ceased in flask 5.

Flasks 1, 2, and 3 each contained the complex salt solution. The only difference among these three flasks during the first 7 36-hour periods was the amount of rumen ingesta inoculum added at the beginning of the first period. Flask 3 contained 70 ml of rumen liquid or half the standard amount; flask 2 had the regular 140 ml of inoculum; and flask 1 had double the standard amount, or 280 ml. Following the initial period, each of these flasks was inoculated in a manner similar to all other flasks by using one-half of the preceding fermentation residue.

Cellulose digestion in each of the flasks containing the complex salt solution was materially better than that observed in flasks in which the complex mixture was omitted (table 1). Comparing flasks 1, 2, and 3 with one another, flask 3 (containing the least amount of original rumen liquid) digested cellulose for a shorter period than the flasks containing larger

TABLE 1

The influence of a complex salt solution and ash of alfalfa extract on in vitro cellulose digestion by rumen microorganisms

PERIOD	FLASK 6 $(NH_4)_2SO_4$ only		FLASK 5 $(NH_4)_2SO_4$ + alfalfa ash		FLASK 1 [1] Complex salt		FLASK 2 [2] Complex salt		FLASK 3 [3] Complex salt		FLASK 4 Complex salt + alfalfa ash	
	CELLULOSE											
	At start	Digested	At start	Digested	At start	Digested	At start	Digested	At start	Digested	At start	Digested
	gm	%	*gm*	%	*gm*	%	*gm*	%	*gm*	%	*gm*	%
1	1.04	68	1.04	81	1.13	77	1.04	89	0.98	89	1.04	86
2	1.12	9	1.05	24	1.08	41	1.01	33	1.01	57	1.02	37
3	1.46	6	1.35	7	1.27	56	1.29	45	1.17	49	1.27	44
4	1.64	—2	1.58	0	1.23	75	1.31	16	1.25	1	1.31	77
5	1.79	2	1.74	2	1.10	7	1.50	34	1.57	2	1.10	64
6	1.82	—2	1.81	2	1.47	10	1.45	9	1.72	5	1.15	29
7	1.89	2	1.84	0	1.61	15	1.61	4	1.77	—2	1.36	19
8					1.63	—6	1.73	24 [4]	1.85	1 [5]		
9					1.82	7	1.61	44	1.87	25		
10					1.80	—2	1.40	61	1.66	45		
11					1.87	—3	1.22	28	1.41	51		

[1] Flask 1 was inoculated with 280 ml rumen liquid or double the usual starting inoculum.
[2] Flask 2 was inoculated with the regular 140 ml of rumen liquid.
[3] Flask 3 was inoculated with 70 ml of rumen liquid, or one-half the standard inoculum.
[4] Beginning with period 8, flask 2 was supplemented with 150 ml of autoclaved rumen liquid.
[5] Beginning with period 8, flask 3 was supplemented with 150 ml of autoclaved extract of manure.

amounts of the original rumen liquid. Despite the improvement noted in all flasks containing the complex salt solution, these flasks also gradually lost their ability to digest cellulose efficiently during or before the 7th 36-hour period.

Fermentation flask no. 4 contained both the complex salt solution and additions of the ash of alfalfa juice extract. As can be seen in table 1, cellulose digestion increased more and persisted for a longer time in this flask as compared with the best digestion occurring in any of the other vessels.

At the end of the 7th 36-hour period all flasks were discontinued except 1, 2, and 3. Since each of these three flasks had almost ceased to digest cellulose, recovery experiments were begun in an effort to restore efficient cellulose breakdown. Flask 1 served as a control to which no special additions were made. To flask 2 were added 150 ml of autoclaved rumen liquid, and to flask 3 150 ml of autoclaved extract of manure, from the 8th through the 11th fermentations.

No cellulose digestion occurred (table 1) in control flask 1 from period 8 through period 11. Conversely, the addition of autoclaved rumen liquid to flask 2 resulted in progressive increases in cellulose breakdown from the 8th through the 10th 36-hour periods. Recovery in ability to digest cellulose was also noted in flask 3, which had the manure liquid additions. The recoveries in both these flasks approached without equaling the maximum cellulose digestion observed earlier in these same flasks when they were originally inoculated with rumen microorganisms, 16 days previously.

In general, the Gram stains observed indicated a close correlation between the size and numbers of organisms present in a given fermentation and the actual cellulose digestion taking place. When conditions were favorable for good cellulose digestion, the Gram stains indicated good bacterial development. Conversely, when cellulose digestion was poor the Gram stains indicated poor bacterial development both as to numbers and as to size.

Some of the larger types of protozoa failed to survive throughout the entire series of fermentations. The pre-

dominating flora at the outset consisted of Gram-negative fat rods, Gram-negative short rods or ovoid organisms with a lesser number of Gram-positive large cocci and long rods. The smaller number of Gram-positive organisms observed may have been due in part to the limitations of the Gram stain technique. The sampling with a wire loop and the washings which accompanied the staining procedure tended to exclude the fibrous material with which the Gram-positive organisms were most often associated.

The predominating types of bacteria present as the fermentation periods progressed appeared to be similar to those originally observed. The most noticeable change in flora occurred in flasks promoting poor cellulose digestion. This change represented both a decreased population and a decrease in individual size of bacteria without any noticeable change in the predominating type of organisms present. In the recovery experiments in which cellulose digestion progressively improved, the size and numbers of bacteria increased, with apparent constancy in the predominating types of organisms observed.

Experiment 2

The cellulose digestion values obtained in experiment 2 are presented in table 2. Four of the 6 flasks — 3, 4, 5, and 6 — during the first 6 fermentation periods were primarily repetitions of those for the first experiment. The results obtained with these flasks approximated those obtained in the earlier experiments. Without the complex salt solution (flask 6) the initial digestion was fairly successful; however, in succeeding periods it dropped off sharply, with no digestion occurring beyond the third day.

The addition of the complex salt solution (flasks 4 and 5) resulted in a higher initial cellulose breakdown, which persisted for a longer time than that occurring where the salt solution was omitted. Flasks 4 and 5 were treated similarly during the first 6 fermentations except for the original inocu-

TABLE 2

Further observations upon the influence of minerals and autoclaved fractions of rumen liquid and manure extract upon cellulose digestion by rumen microorganisms

PERIOD	FLASK 6 $(NH_4)_2SO_4$ only		FLASK 4 Complex salt		FLASK 5 Complex salt [1]		FLASK 3 Complex salt + alfalfa ash		FLASK 1 Complex salt + auto. rumen liquid		FLASK 2 Complex salt + auto. extract of manure	
	CELLULOSE											
	At start	Digested	At start	Digested	At start	Digested	At start	Digested	At start	Digested	At start	Digested
	gm	%	*gm*	%	*gm*	%	*gm*	%	*gm*	%	*gm*	%
1	1.01	60	1.01	92	1.01	64	1.01	88	1.01	86	1.01	92
2	1.14	37	0.98	68	1.12	79	1.00	74	1.02	48	0.99	86
3	1.30	1	1.10	72	1.06	42	1.07	82	1.22	74	1.01	83
4	1.58	0	1.09	4	1.25	14	1.04	58	1.11	63	1.03	87
5	1.73	—1	1.47	0	1.48	0	1.16	60	1.15	49	1.01	94
6	1.81	0	1.67	—1	1.68	0	1.17	61	1.24	17	0.97	90
7	1.84	—1 [2]	1.78	0 [2]	1.79	8 [2]	1.17	54 [2]	1.47	24	0.99	77
8	1.87	—1	1.83	—5	1.77	31	1.21	17	1.51	40	1.06	76
9	1.89	—1	1.90	4	1.56	50	1.44	2	1.40	11	1.07	90
10	1.89	1	1.85	3 [4]	1.34	64	1.65	5 [4]	1.57	0	0.98	36
11	1.87	—1	1.84	—2	1.18	50	1.72	4	1.74	0	1.27	29
12	1.88	—4	1.88	0	1.24	64	1.77	2	1.82	1	1.39	41
13	1.92	3	1.88	0	1.17	82	1.81	—1	1.85	—3	1.35	50

[1] Flasks 4 and 5 during the first 6 36-hour periods differed only with respect to the original starting inoculum. Flask 4 received the standard 140 ml of ingesta liquid, while flask 5 received the heavy portion centrifuged from 140 ml ingesta liquid.

[2] Flasks 6, 4, and 3 received equivalent amounts of urea nitrogen in place of ammonium sulfate beginning with period 7.

[3] Flask 5 received 150 ml of autoclaved and centrifuged extract from cow manure beginning with period 7.

[4] The following materials (parts per million of fermentation contents) were added to flasks 3 and 4 beginning with period 10: thiamine, 0.1; riboflavin, 0.1; niacin, 0.1; calcium pantothenate, 0.1; pyridoxine, 0.1; biotin, 0.0004; para-aminobenzoic acid, 0.1; inositol, 0.1; vitamin B_{12}, 0.0004; adenine, 10; guanine, 10; uracil, 10; xanthine, 10; tryptophan, 100; methionine, 100; lysine, 100; threonine, 100; leucine, 100; histidine, 100; valine, 100; and phenylalanine, 100.

lum used; flask 4 received the standard 140 ml of ingesta liquid, whereas 5 received the heavy material centrifuged (3,000 R.P.M.) out of 140 ml of ingesta liquid. This heavy material represented 10% of the 140 ml, or 14 ml.

Alfalfa juice ash added along with the complex salt solution (flask 3, see table 2) again led to improvement in cellulose digestion over that occurring in flasks containing only the salt solution. This flask continued to digest cellulose very satisfactorily for 10 days but subsequently failed to do so.

Various recovery trials were attempted with flasks 3, 4, 5, and 6 beyond the 6th fermentation. The addition of autoclaved manure extract to flask 5 was highly successful. Recovery in ability to digest cellulose was progressive with each of the first 4 additions, apparently being complete with the 7th addition.

Equivalent amounts of urea nitrogen were substituted for the ammonium sulfate nitrogen in flasks 3, 4, and 6 after the 6th fermentation with no appreciable effects.

A mixture of B vitamins, amino acids, and other bacterial growth substances (table 2) was added to flasks 3 and 4 after the 9th 36-hour period. No favorable influence could be noted from these additions; however, more observations need to be made before conclusions can be drawn as to their lack of essentiality.

In flasks 1 and 2 attempts were made to maintain cellulose digestion at a high plane throughout the entire series of fermentations by adding respectively autoclaved rumen liquid and autoclaved manure extract as was done with the additions made in recovery trials. The rumen liquid additions were only partially successful; the manure extract additions were much more so. Good cellulose digestion, averaging close to 90%, occurred throughout the first 9 periods, followed by a decreased but fairly successful digestion during the last 4 periods.

The Gram stains observed in the second experiment followed the same general pattern noted in the first experiment,

with one exception. This occurred in flask 1, containing the autoclaved rumen liquid additions. Despite the lack of cellulose digestion in this flask during the 10th through the 13th 36-hour fermentations, the microflora appeared to be as numerous and as well developed as was noted earlier when cellulose digestion was quite active. The explanation for this condition is not evident at present.

In general, however, good cellulose digestion was accompanied by good bacterial development. Poor cellulose digestion was correlated with smaller numbers of microorganisms and a decrease in their individual size.

DISCUSSION

The results obtained in this study indicate clearly that the microflora found in the rumen of cattle are capable of breaking down cellulose to a high degree when favorable growth conditions are present. Much more experimental data must be accumulated before these favorable conditions can be fully enumerated and defined with a high degree of accuracy. Nevertheless, it appears worth while to consider some of the general factors which seem to be involved in microbiological digestion both in the laboratory and in the live animal.

For convenience, these factors can be arbitrarily divided into two groups. The first represents those conditions which are more or less fixed or regulated by the physiology and anatomy of the host animal. The second group of factors represents influential conditions subject to variations related directly to the environment of rumen microorganisms or indirectly to the environment of the host animal. Such factors as temperature, moisture, the salts contained in saliva, anaerobiosis, absorption of organic acids through the rumen wall, motion or stirring, and possibly the exclusion of light, would fall in the first group. Influential factors in the second group include the types of microorganisms present in the rumen or in the inoculum of the fermentation flask; the nutrients avail-

able to the bacteria present; and certain properties of the medium such as pH, total acidity, oxidation-reduction potential, total salt concentration, and possibly symbiotic relations between the different types of organisms present.

The need for maintaining each and all of these favorable microbiological influences receives added emphasis when it is recalled that the reaction or digestion being studied must take place at a very rapid rate. Feeds entering the rumen of cattle must be acted upon in periods averaging no longer than 24 hours (Burroughs et al., '46). This is an extremely short period for fiber digestion when such digestion is compared with other forms of microbiological decomposition, such as the breakdown of organic matter in the soil, sewage diposal, and industrial fermentations of fibrous materials. A slightly longer period of fermentation was used in the laboratory studies as compared with what actually occurs in the live animal, because the best initial starting conditions in *in vitro* experiments are likely to be inferior to *in vivo* conditions.

The temperature of the rumen fosters a rapid rate of fermentation. It varies little in different cattle and throughout a 24-hour day. Continuous recorded temperatures in the rumen of cattle with rumen fistulae (unpublished data) gave an average value of 40°C.

The moisture content of the rumen also favors rapid fermentation. This is maintained by water consumed by the animal and the large quantity of saliva which continuously flows into the rumen. The saliva, in addition to furnishing moisture, has high buffering action and may also contribute to the mineral nutrition of microorganisms. In cattle, the moisture content in the rumen usually varies between 85 and 90% (Burroughs et al., '46). In the fermentation flasks studied in the laboratory the moisture content exceeded even these high values. It was purposely made high to avoid injurious effects from the accumulation of organic acids (Buswell and Hatfield, '34). In the rumen the breakdown or removal of these acids offers less of a problem than in a glass fermentation vessel,

since acids are absorbed through the rumen wall. A diffusible bag can be used instead of a glass fermentation flask in partially getting rid of organic acids; however, in preliminary trials, these bags proved cumbersome to handle in long-time fermentations and were subject to rupture.

The importance of anaerobic conditions to rumen microorganisms is paramount. The rumen is primarily an anaerobic chamber, except for a short time following feed consumption (Washburn and Brody, '37). Gall et al. ('47) pointed out the importance of anaerobiosis in culture studies with rumen bacteria.

The favorableness to cellulose digestion of motion or stirring within the fermentation flask is unknown. Certainly within the rumen itself a great deal of stirring takes place resulting from the rhythmical contractions of the rumen muscular pillars, the animal's locomotion, and the activty of motile protozoa. Some advantage seemed to accrue at times from the occasional rapid passage of carbon dioxide through the glass fermentation vessels. It is not certain, however, that this improvement was the direct result of stirring or of the more rapid washng out of accumulated organic acids.

The above favorable conditions for the microbiological digestion of cellulose, which are more or less fixed or regulated by the physiology and anatomy of the host animal, are primarily of academic interest with respect to their functions in cattle and other ruminants. Their major interest would seem to lie in the need for controlling or duplicating such conditions in attempting to study rumen fermentation experimentally outside the live animal. Of far more practial interest to the animal physiologist are the conditions favorable to rumen digestion which are subject to environmental variations, such as the types of rumen organisms present and the nutrient or other needs of such organisms. These conditions are subject to alteration through livestock feeding and management and may be of considerable economic importance in livestock production.

The finding or development of superior types of rumen organisms represents a fertile field for inquiry. Gall et al. ('49) reported the types and numbers of rumen bacteria found in mature cattle and sheep fed rations of a widely different nature. This work indicated differences in the predominating types of rumen microorganisms present under different feeding regimes; however, these differences were more quantitative than qualitative in nature. That is, approximately the same general types of organisms were present in each animal even though the predominating type was very different.

The selection of inocula from different mature animals for use in *in vitro* cellulose digestion studies may not be a major problem if further investigations indicate an approximate qualitative constancy in rumen microflora. Pounden and Hibbs ('48), however, presented evidence that young calves (immature ruminants) reared in quarters isolated from mature animals and fed rations low in roughage develop rumen microflora which differ materially from those found in calves reared under more natural conditions.

Mention should be made of the size of the large starting inoculum taken from the rumen as well as of the large inoculum used in subsequent fermentation periods. These inocula are essential to accelerated rates of fiber digestion. In the ruminant they probably constitute a factor even more important than in the fermentation occurring in flasks. The unfortunate part of using large rumen inocula in experimental flasks is the introduction of large quantities of unknown constituents aside from the microorganisms present. The laboratory procedure of using only half the previous fermentation residue in the succeeding fermentation tends to offset this unfortunate condition, in that the original material taken from the rumen is progressively diluted by the introduction of new materials at the start of each 36-hour period.

The nutrients available in and other characteristics of the feed and water entering the rumen or contained in the fermentation medium probably exert the major influences upon

cellulose breakdown by rumen microorganisms. The present method of studying these influences in the laboratory offers an aid for their quantitative measurement, many of them being most difficult if not impossible to measure in the intact live animal. The limited amount of data in this paper suggests that minerals from different sources are beneficial to rumen microorganisms in digesting fiber. This tends to confirm the beneficial influence of the ash of alfalfa upon roughage digestion which was observed in the *in vivo* experiments with cattle referred to earlier.

The recovery experiments also indicated that substances in autoclaved rumen liquid and autoclaved extract from manure were beneficial in rumen microbiological digestion of cellulose. The detailed nature of these beneficial influences requires further investigation.

SUMMARY

A laboratory method is described for studying cellulose digestion by rumen microorganisms. The major features of this method include the use of continuous 36-hour fermentation periods, in which the original starting inoculum was a mixed culture of organisms taken directly from the rumen of cattle. The inoculum used in the second and succeeding 36-hour periods consisted of half the residue from the preceding fermentation. Cellulose digestion was determined chemically on the half portions not used as inoculum, and Gram stains of the microflora were observed at the beginning and end of each fermentation period.

Application of the method in preliminary trials resulted in data showing pronounced differences in the ability of rumen microorganisms to digest cellulose, depending upon additions or withdrawals from the nutrient medium as the fermentation periods progressed. Additions which proved helpful to cellulose digestion included a complex salt solution, the ash of alfalfa extract, autoclaved rumen liquid, and an autoclaved water extract of manure. Recovery trials with certain addi-

tions of these materials restored the ability to digest cellulose which had previously been lost through the omission of such additions.

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BIOLOGICAL VALUES OF SIX PARTIALLY PURIFIED PROTEINS FOR THE ADULT ALBINO RAT

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ONE FIGURE

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A cooperative study of the nutritive value of proteins was initiated by the Bureau of Biological Research of Rutgers University in 1946 for the purpose of comparing methods of assay involving maintenance, maintenance and growth, or regeneration of specific tissue proteins. A comparison of protein utilization by various species was also planned, involving the mouse, the rat, the dog, the chicken and human subjects. Six protein sources were selected to represent a variety of patterns of amino acid composition of interest in nutrition. Quantities of these 6 proteins, or high-protein foods, were distributed among some 30 or more laboratories, either for biological assay by specified procedures or for amino acid analysis. This laboratory was invited to assay the selected proteins or protein foods for their digestibility and biological value for maintenance in the adult albino rat, for which we used the nitrogen balance method previously described by Bricker, Mitchell and Kinsman ('45).

The 6 protein sources, with the maximum processing temperatures, were dried egg albumin (65.5°C.), desiccated and defatted whole egg (below 70°C.), dried and defatted beef muscle (82.2°C.), wheat gluten (60°C.), casein (60–70°C.), and peanut flour (no temperatures noted). The preparation of these test products is fully described in an addendum to

the article by Hawley, Murlin, Nasset and Szymanski ('48) reporting results obtained with these 6 protein foods in adult human subjects.

The present paper reports the results secured with growing and with adult rats; these will be compared with the results on the same proteins reported by the University of Rochester group with adult men and women. The test proteins in the latter experiments were served in a cooked form, except for one sample of casein. In the rat experiments the test proteins were consumed in the form in which they were received. This discrepancy in the method of feeding the test proteins introduces an element of uncertainty in the comparison of rats and humans. However, judging from experiments on rats, the baking of proteins in the presence of water for 40 minutes at 350°F. (Hawley et al., '48) would not be expected to affect the biological value appreciably (Block and Mitchell, '46). The digestibility of the cooked products generally exceeded 98% (see table 5).

EXPERIMENTAL PROCEDURES

The moisture, ether extract and conventional protein ($N \times 6.25$) contents of the test products are summarized in table 1. The products were made up into experimental diets containing approximately 1.5 to 5.0% of protein, depending upon the expected nutritive value; the purpose being to prepare two rations containing two protein levels for each test food selected, so that both levels would be associated with negative nitrogen balances, or, at most, balances not greatly positive. All diets were equalized in fiber content at 2% and in fat content at 12%, and were adequately provided with all minerals and vitamins known to be required by the rat.

Some comparative tests in connection with the present work were undertaken with growing rats by the nitrogen balance method (Mitchell et al., '45). In these cases the protein level was raised to approximately 10%, but otherwise the diets were designed in the same way as the present

ones for mature rats. The carbohydrate components of all diets consisted of glucose, dextrin and sucrose; and the fat, other than that provided by the test food, consisted of lard with small amounts of wheat germ oil (0.5%) and cod liver oil (1.5%).

TABLE 1

Chemical composition of protein samples

TEST PROTEIN	MOISTURE	ETHER EXTRACT	PROTEIN (N × 6.25)
	%	%	%
Dried defatted whole egg	5.18	0.19	76.75
Dried defatted beef muscle	2.60	0.35	98.31
Casein	8.68	0.16	89.19
Dried egg albumin	7.97	0.15	81.56
Wheat gluten	7.70	0.70	88.00
Peanut flour	2.42	5.10	62.56

The mature rats used in these experiments were all males, and with few exceptions were discarded breeders obtained from dealers.[1] They ranged in weight from 245 to 490 gm. They were handled in groups of 8 or 10, each group consisting of 4 or 5 pairs of rats matched as closely as possible for initial weight and fed equal amounts of their respective diets throughout their respective experiment. A typical experimental plan is the following:

	PERIOD 1	PERIOD 2	PERIOD 3
5 rats	4% test protein	standardizing diet	2% test protein
5 rats	2% test protein	standardizing diet	4% test protein

In some tests, however, one level of protein only was tested in period 1 and the other in period 2.

The standardizing diet was similar to the test diets except that it contained approximately 3% of whole egg protein prepared in this laboratory. In a number of the tests the standardizing period was preceded or followed by a period

[1] Fifty-seven rats, ranging in weight from 350 to 450 gm, were secured from Sprague-Dawley of Madison, Wisconsin. Six rats, ranging from 240 to 298 gm in weight, were obtained from the Harlan Small Animal Industry, Cumberland, Indiana.

on a practically nitrogen-free diet, containing no deliberately incorporated protein source. The animals in all periods were fed 12 gm of their respective diets daily, moistened to prevent scattering.

A typical plan of experiment for the growing rats, which weighed initially 50 to 60 gm, follows:

PERIOD 1	PERIOD 2	PERIOD 3
5 rats 10% wheat gluten protein	standardizing diet	10% peanut protein
5 rats 10% peanut protein	standardizing diet	10% wheat gluten protein

In the experiments with these growing rats, the standardizing diet contained 4% of whole egg protein prepared in the laboratory.

In all feeding experiments the collection periods were of 7 days' duration, with prefeeding periods of the same length during which the diet was constant. Feces markers were used.

The biological samples were analyzed for nitrogen by the macro-Kjeldahl method, using mercury as a catalyst. The food and feces samples were digested 4 hours after clearing and the urine samples one hour.

The results secured on the growing rats were analyzed by the usual method developed in this laboratory, designed to give coefficients of true digestibility for the nitrogen and biological values representing the percentage of the absorbed nitrogen retained for maintenance and growth.

The data for the mature rats were interpreted by plotting, for each protein source, the amount of absorbed nitrogen in the three experimental periods for all rats against the nitrogen balance, both expressed to the basal calorie, estimated at 686 cal. per m^2 per day (see Bricker and Mitchell, '47). The surface area was estimated by the formula of Lee ('29). The data for the standardizing periods were considered as representing the results of complete nitrogen inanition, since the egg N contained in the standardizing diet made insignificant contributions to the urinary and fecal nitrogen. A linear equation was fitted to the pooled data for each protein source. The slope of this line ($\times 100$) is the biological value of the protein. The true digestibility was computed in the usual

way by making correction for the metabolic fecal N as measured per gram of air-dry food in the standardizing period.

EXPERIMENTAL RESULTS

The mature rats generally lost in weight throughout the experiment, the rate of decline depending on the nature of the protein source and the level of protein feeding. On the better proteins at the higher levels the weight loss averaged less than 1 gm a day. On the poorer proteins at the lower levels the weight loss averaged up to 2 gm a day.

The data secured with the mature rats are presented in figure 1, which shows graphically the relationship between the nitrogen balance and the absorbed nitrogen, both expressed per calorie of basal heat. This relationship seems to be a linear one, and therefore the equation of a straight line was fitted to each set of data by the method of least squares; the resulting equations appear in the figure. The symbols $\overline{AN}$ and $\overline{NB}$ represent, respectively, the absorbed nitrogen and the nitrogen balance, which are given in milligrams per basal calorie. The constant term on the right hand side of the equations represents the nitrogen balance on a nitrogen-free diet: presumably the sum of the minimum endogenous nitrogen and the metabolic fecal nitrogen in milligrams per basal calorie. The coefficient of $\overline{AN}$ is the rate of increase of the nitrogen balance per unit increase in the absorbed nitrogen. When multiplied by 100 it gives the biological value of the protein in replacing endogenous losses and the metabolic fecal loss.

In table 2 are assembled the biological values of the 6 test proteins with their standard errors, computed according to Rider ('39). Statistical analysis of the significance of differences between biological values determined by this method will be found in table 3. From these calculations it will be noted: (a) that the proteins of egg albumin are shown to be definitely superior in metabolic utilization to all other test proteins except whole egg proteins; (b) that the proteins

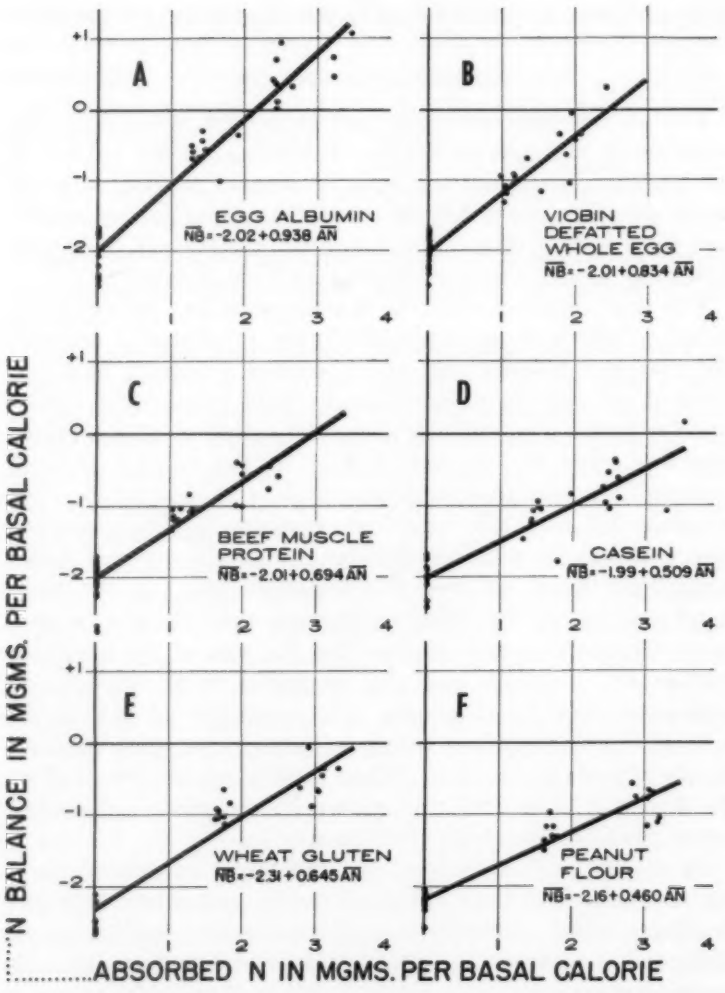

Fig. 1 The relationship between the nitrogen balance and the absorbed nitrogen, both expressed in milligrams per basal calorie, for the 6 test proteins.

of peanut flour proved definitely inferior to all proteins but casein; and (c) that the proteins of wheat gluten, while definitely inferior in biological value to those of egg albumin and of whole egg, and definitely superior to the proteins of

TABLE 2

The biological values of the test proteins for the mature rats

PROTEIN SOURCE	BIOLOGICAL VALUE WITH STANDARD ERROR	PROTEIN SOURCE	BIOLOGICAL VALUE WITH STANDARD ERROR
	%		%
Egg albumin	93.8 ± 1.6	Wheat gluten	64.5 ± 1.6
Whole egg (commercial)	83.4 ± 1.8	Casein	50.9 ± 1.5
Whole egg (commercial)[1]	81.6 ± 4.2	Peanut flour	46.0 ± 1.3
Beef muscle	69.4 ± 1.2		

[1] This is a repeat determination on the same batch of dried and defatted whole egg, using 9 mature rats. The data for this test are not included in figure 1.

TABLE 3

Statistical analysis of the differences between biological values listed in table 2

PROTEIN SOURCES		WHEAT GLUTEN	CASEIN	EGG ALBUMIN	PEANUT FLOUR	BEEF MUSCLE	WHOLE EGG
Wheat gluten	t =		2.064	4.222	3.223	0.872	2.779
	P =		0.046	< 0.001	0.004	0.39	0.009
Casein	t =	2.064		6.076	0.807	3.136	4.590
	P =	0.046		< 0.001	0.425	0.005	< 0.001
Egg albumin	t =	4.222	6.076		7.411	3.942	1.400
	P =	< 0.001	< 0.001		< 0.001	< 0.001	0.17
Peanut flour	t =	3.223	0.807	7.411		4.579	6.032
	P =	0.004	0.425	< 0.001		< 0.001	< 0.001
Beef muscle	t =	0.872	3.136	3.942	4.579		2.280
	P =	0.39	0.005	< 0.001	< 0.001		0.03
Whole egg[1]	t =	2.779	4.590	1.400	6.032	2.280	
	P =	0.009	< 0.001	0.17	< 0.001	0.03	

[1] Disregarding the repeat determination cited in table 2.

peanut flour and to casein (at the 5% level), are not to be distinguished statistically from the proteins of beef muscle.

The nitrogen utilization data on 4 of the test proteins, secured with the growing rats, are summarized in table 4.

It will be noted in particular that, for maintenance and growth, the proteins of wheat gluten are definitely inferior to the proteins of peanut flour, while for maintenance alone in the adult rat they are markedly and definitely superior to peanut proteins (see table 3).

TABLE 4

Utilization of 4 of the test proteins by young growing rats

RAT NUMBER	WHEAT GLUTEN		PEANUT FLOUR		RAT NUMBER	EGG ALBUMIN		WHOLE EGG	
	Digestibility (corr.)	Biological value	Digestibility (corr.)	Biological value		Digestibility (corr.)	Biological value	Digestibility (corr.)	Biological value
1	100	42	98	55	11	100	97	99	91
3	100	46	98	55	13	100	97	98	90
5	97	38	96	58	15	100	96	97	88
7	100	38	97	56	17	100	94	98	92
9	98	32	97	59	19	100	96	98	92
2	100	45	96	43	12	100	98	98	78
4	99	40	96	52	14	100	100	96	82
6	98	42	96	65	16	100	98	95	84
8	100	39	96	48	18	100	98	97	84
10	99	39	97	51	20	100	99	97	89
Averages	99.1	40.1	96.7	54.2		100	97.3	97.3	87.0
S. E.		± 1.3		± 1.9			± .54		± 1.5

DISCUSSION

The whole egg protein submitted for test was assayed for digestibility and biological value against a standardizing diet containing 3% of protein supplied by a sample of whole egg that had been dehydrated and defatted in this laboratory. At the same level of intake the nitrogen of the test product proved to be less digestible and less utilizable in metabolism than that of the laboratory product, when tested on adult rats; the true digestibility of the nitrogen of the test product was 98% and the biological value 82, taking the values for the laboratory product to be 100 in each case.

For growing rats, also, the whole egg protein submitted for test proved to be less available, both in digestion and in metabolism, than that of the laboratory product. Using two

groups of 5 young rats each, the values for the test and the laboratory whole egg, both fed at a 10 to 10.5% protein level, were, respectively, 97.6 and 98.6 for coefficients of true digestion, and 86.8 and 97.6 for biological value. The difference in digestion coefficients is not clearly significant statistically, with a "t" value of 2.13 and a probability of a chance outcome of 0.07. The difference in biological value, however, is highly significant, "t" being equal to 12.07 where a value of 3.35 is equivalent to a probability of 0.01.

In some of the tests the effect on the nitrogen metabolism of mature rats of a diet containing 3% of conventional protein from the laboratory preparation of whole egg, and of a diet as nearly nitrogen-free as was possible, was determined. From data on 35 rats fed each of these diets in contiguous periods, the following averages, with their standard errors, were secured:

DIET	URINARY N per $W^{.75}$ gm, mg/day	FECAL N per gram of food, mg
3% lab. whole egg protein	0.554 ± 0.015	1.45 ± 0.034
N-free	0.590 ± 0.012	1.24 ± 0.024
Difference	0.036 ± 0.019	0.21 ± 0.042

Since each of the 35 rats in this comparison received in adjacent periods each of the two rations, it is possible to make a paired comparison of the data by the method of Student ('25). The differences in urinary and fecal excretions of nitrogen between the N-free and the whole egg protein diet proved to be highly significant at a probability level of much less than 1%. Although significant statistically, the differences are not great in magnitude. The difference in urinary nitrogen amounted to 6.3%, which cannot be attributed to a time effect in the attainment of the minimum endogenous nitrogen output, since half of the rats received the N-free diet first and the other half received the 3% whole egg protein diet first. Its explanation may well be that whole egg protein is somewhat more efficient in replacing the en-

dogenous losses and the metabolic fecal losses of nitrogen than the tissue proteins which, in the absence of proteins in the food supply, are raided to maintain the concentration of nitrogenous tissue constituents performing some more vital functions in the body.

The difference in the fecal output of nitrogen, assuming that on the nitrogen-free diet to be entirely of metabolic origin, is equivalent to a true digestibility of 97.6% for the whole egg nitrogen.

TABLE 5

The biological value and true digestibility of the test proteins for growing rats, mature rats and adult men

PROTEIN	BIOLOGICAL VALUE			TRUE DIGESTIBILITY		
	Growing rats	Mature rats	Mature humans [1]	Growing rats	Mature rats	Mature humans [1]
Egg albumin	97	94	91	100	96	101
Whole egg (commercial)	87	82	94	97	98	98
Beef muscle	76 [2]	69	67	100 [2]	99	97
Wheat gluten	40	65	42	99	98	104
Casein	69 [2]	51	56	99 [2]	96	91
Peanut flour	54	46	56	97	95	99

[1] Taken from Hawley, Murlin, Nasset and Szymanski ('48).

[2] Taken from Block and Mitchell ('46).

In table 5 the biological values and coefficients of digestion of the nitrogen in the 6 protein sources for growing rats, for mature rats and for humans are assembled for comparison. The differences in digestibility are not great, with one or two exceptions, and their discussion, therefore, does not seem to be profitable.

The differences in biological value, however, may be correlated to some extent with the amino acid composition of the proteins. Beef muscle, casein and peanut flour are deficient in cystine or methionine or both for the growing rat, while wheat gluten is markedly deficient in lysine (see Block and Mitchell, '46). Whole egg protein, when prepared with minimum damage, is not improved in growth-promoting value for the rat, in our experience, by supplements of any one of

the essential amino acids with the possible exception of lysine, which needs further study. In table 5 it will be noted that for those proteins deficient in cystine-methionine the biological values are lower for the adult rat than for the growing rat and, with the exception of beef muscle (in which the cystine-methionine deficiency is probably slight) they are lower than for the adult human. On the other hand, for wheat gluten, deficient in lysine, the value for the adult rat is considerably higher than for the growing rat or for the adult human. These relationships indicate that the cystine-methionine requirement is relatively more intense for the adult rat than for either the growing rat or the adult human. On the other hand, the lysine requirement seems to be much less prominent among the amino acid requirements of the adult rat than among those of the growing rat or the adult human, confirming the conclusion recently expressed by the senior author of the present paper (Mitchell, '47).

The utilization of proteins in adult metabolism may be expressed, and compared among different species, as the amount of absorbed nitrogen per basal calorie required for nitrogen equilibrium. The justification for this method of expression is the Terroine-Smuts demonstration (see Smuts, '35; Mukherjee and Mitchell, '49) that the maintenance requirement of protein in adult animals of different species varies with the basal metabolism, though the ratio of endogenous nitrogen loss to basal metabolism may be disturbed by differences in age, sex, prior treatment, and so forth (Treichler and Mitchell, '41). The desired values may be obtained from the data of this experiment by solving for $\overline{AN}$ when $\overline{NB} = 0$ in the equations for the different proteins which express the relationship between the absorbed nitrogen and the nitrogen balance in milligrams of nitrogen per basal calorie. In the 6 equations given in figure 1 the constant term varies, presumably due to an error of sampling since there is no reason to suspect a correlation between the output of nitrogen on a nitrogen-free diet and the biological value, the coefficient of AN. Hence, in computing the values

for the adult rat given in table 6, the average of all these constants, i.e., — 2.16, has been used, with the assurance that the values so computed would be more nearly comparable. The values for the adult human were computed in an analogous fashion from the data of Hawley et al. ('48), supplemented by further necessary information kindly provided by Dr. J. R. Murlin. The table also contains data from other sources, as is revealed in the footnotes.

The protein values expressed as milligrams of nitrogen per basal calorie required for nitrogen equilibrium reveal again the more intense requirement of the adult rat for

TABLE 6

The requirements of absorbed nitrogen from different sources for nitrogen equilibrium by adult rats and adult humans

PROTEIN SOURCE	ABSORBED N REQUIRED FOR N EQUILIBRIUM	
	Adult rat	Adult human [1]
	mg N per basal cal.	
I. *Proteins of unknown deficiencies*		
Whole egg, laboratory prep'n.[2]	2.16	...
Whole egg, commercial	2.63	2.08
Egg albumin	2.30	2.14
II. *Proteins deficient in cystine-methionine*		
Beef muscle	3.11	2.93
Casein	4.24	3.31
Peanut flour	4.70	3.51
Soy flour [3]	5.48	2.65
Milk [3]	3.18	2.59
III. *Proteins deficient in lysine*		
Wheat gluten	3.20	4.67
White flour	3.32 [4]	4.61 [3]

[1] Unless otherwise indicated, these data were computed from the results of Hawley et al. ('48), with the aid of additional information kindly supplied by Dr. J. R. Murlin.

[2] This is the average excretion of N on a N-free diet of the adult rats in this experiment. (See immediately above.)

[3] The rat data are taken from Bricker and Mitchell ('47), the human data from Bricker, Mitchell and Kinsman ('45).

[4] Computed from a biological value of 65 (Mitchell, '47) and the value of 2.16 mg of N excreted per basal calorie on a N-free diet.

cystine-methionine, and the more intense requirement of the adult human for lysine. Mueller and Cox ('47) have shown that for adult man casein and lactalbumin are of equal value as sources of amino acids; the cystine-methionine deficiency of casein for the rat does not limit its value for the human. On the other hand, the lysine deficiency of white flour proteins is not in evidence in adult rodent nutrition (Mitchell, '47), although readily demonstrable in adult human nutrition (Bricker, Mitchell and Kinsman, '45). These results are not opposed to the observation of Wissler et al. ('48) that lysine is required by the adult rat for nitrogen equilibrium. The evidence obtained in the present experiment, as well as in those cited by Mitchell ('47), shows merely that the lysine requirement is relatively small in the adult rat.

The intense cystine-methionine requirement of the adult rat and the low requirement for lysine may be explained on the basis of the high cystine and low lysine content of hair (Block and Bolling, '45) and the probability that the amino acid requirements of the adult rat are dominated by the requirements for hair growth. The importance of hair growth in determining amino acid requirements would be much less for the growing rat, in which the most active protein growth relates to other tissues than hair, and also much less for the adult human, whose body is only sparsely covered with hair. The lysine content of hair ranges from 2 to 3% per 16 gm of nitrogen, being similar in this respect to the proteins of wheat gluten (2.0%) and other cereal products (Block and Mitchell, '46). On the other hand, muscle contains 8% of lysine or more on the same basis.

The argument just presented assumes that hair growth will continue during periods when the animal is in negative nitrogen balance, since the experiments on adult subjects were carried out when the subjects were in this condition. In support of this assumption, it may be pointed out that it seems impossible to denude an animal of hair by long periods of protein depletion; that in the adult rat hair growth is continuous throughout life (Fraser, '31); and that the depressing

effect of cystine or methionine on the endogenous nitrogen output reported for the rat (Brush et al., '47), dog (Allison, Anderson and Seeley, '47), and molting hen (Ackerson and Blish, '25–'26) but not for man (Johnson et al., '47) may be interpreted as an inhibition of an accelerated endogenous protein catabolism initiated mainly in response to a demand for cystine for keratin synthesis in hair (or feather) growth.

SUMMARY AND CONCLUSIONS

The digestibilities and biological values of 6 protein sources of widely different nutritional quality (whole egg, egg albumin, beef, casein, wheat gluten and peanut flour) were determined with adult male albino rats by the nitrogen balance method. Four of these protein sources were also tested with young growing rats. The results have been compared with those secured by Hawley and associates ('48) with adult human subjects, with the same protein sources and by a nitrogen balance method.

The digestibility of the proteins does not differ greatly for growing rats, adult rats and adult humans, but the biological values are quite different for those proteins deficient in cystine-methionine (beef, casein, peanut flour) or in lysine (wheat gluten).

On the basis of this experiment, supported by other data, it may be concluded that the cystine-methionine requirement of the adult rat is more intense, in relation to requirements for other amino acids, than that of the growing rat or of the adult human. On the other hand, the lysine requirement is much less. These differences may be explained on the basis of the amino acid composition of hair and the relative importance of hair growth in the protein nutrition of the growing rat, the adult rat and the adult human.

Except for differences in the proportional requirements for cystine-methionine and lysine, the dietary nitrogen requirement for nitrogen equilibrium is of the same order of magnitude for adult rats and adult humans when expressed as milligrams of nitrogen per calorie of basal metabolism.

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RESURVEY OF NUTRITIONAL STATUS IN NORRIS POINT, NEWFOUNDLAND [1]

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TWO FIGURES

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In June, 1944, a nutrition survey was made of a selected population sample in Norris Point, Newfoundland, a typical fishing and logging village of about 100 families on the west coast of the island (Metcoff et al., '45). The object of the study was to detect the poorest nutrition in the community, and to establish a baseline for future observations. In July, 1948, a resurvey was made of a similar population sample. In the 4 years between the two surveys, the Newfoundland government had initiated a program to improve the nutrition of the population. Among the most important tangible components of the program were, beginning July 1, 1944, the compulsory enrichment of all flour according to U. S. standards and, from 1946, the fortification of margarine with vitamin A at a level of 45 I.U. per gram. The present study was an effort to determine whether detectable changes in nutriture had occurred during these intervening 4 years.

The out-ports (fishing villages) of Newfoundland are particularly suitable for evaluation of measures designed to influence nutrition. The population is static; insularity and

[1] This survey was made possible by a grant from the Williams-Waterman Fund.

terrain make agricultural self-sufficiency impossible; transportation facilities are limited. Nutritional deficiency has long been prevalent in Newfoundland: beriberi has appeared at intervals and several surveys made between 1914 and 1941 indicated a high incidence of vitamin A deficiency; dental caries; spongy, bleeding gums; and chronic malnutrition. Infant mortality, the death rate from tuberculosis, and the incidence of premature and stillbirths have been high. These latter, of course, reflect many influences other than nutrition.

The 1944 survey in Norris Point showed that dental caries, disease of the gums, and findings suggesting deficiency of vitamin A, riboflavin, and iron were common. Adamson and associates ('45) in a larger study made in Eastern Newfoundland reported a high incidence of dental caries, of clinical signs which were attributed to chronic deficiency of vitamin A, riboflavin, ascorbic acid and possibly niacin, and a low urinary excretion of thiamine which, together with subjective symptoms, were interpreted to indicate mild thiamine deficiency.

The nutrition program of the Newfoundland government between 1944 and 1948 included many activities in addition to the compulsory enrichment of flour and margarine. A Nutrition Council, including representatives of the government, the medical profession and other interested groups, was organized in 1945. That summer, Dr. C. P. Cuthbertson of the Medical Research Council (England) studied the nutrition problem in Newfoundland and made recommendations for its solution. In 1946, a nutrition advisor from the Imperial Bureau of Animal Nutrition, United Kingdom, assisted in the organization of a nutrition program. In 1947, a nutritionist was appointed to the staff of the Department of Public Health and Welfare. Pamphlets on nutrition were distributed to teachers and to public health nurses who had to do with the care of expectant mothers and infants. Educational activities included radio talks, press articles, and discussions

before adult education groups. Instruction in nutrition was added to the teachers' training course of Memorial College in St. Johns.

In 1947 a school feeding program was initiated in which a hot drink of reconstituted cocoa-milk powder [2] was supplied daily without cost from October to June. An average of 8½ lb. of this powder per child was distributed yearly. Cod liver oil was distributed free to schools in quantities providing one teaspoonful per child per day for three winter months; it was not administered under supervision. Concentrated orange juice was made available without cost to expectant and nursing women and to young infants. Beginning in November, 1947, all flour entering Newfoundland was reinforced to a level of 0.5% with bone meal containing 31% calcium.

Data on food imports for Newfoundland between 1944 and 1948 showed an increase in the amount of evaporated milk, sugar, jams, and syrups, and a decrease in citrus fruits and juices entering the country, with no definite trend in the imports of other commodities. The economic situation in Newfoundland improved considerably between 1944 and 1948. These alterations are presented in detail by Aykroyd et al. ('49).

The population of Norris Point shared somewhat in this improvement: houses had been painted, a new fish cannery established, and a few more families owned cows. A new road had been constructed from the village across the bay to the only railroad in the country. This facilitated transportation during the summer, although isolation in the winter was unaffected. No improvement in food storage facilities or in agricultural production was apparent. The only change in available foods appeared to be a slight increase in fluid milk, margarine fortified to a higher level with vitamin A, and the replacement of unenriched with enriched flour. Some of the children received cod liver oil. Improved economic

[2] Skim milk powder 70%, sugar 22%, cocoa 8%.

status may have made possible a slightly more liberal diet for some — such an effect was not believed to have been a major one, however.

METHOD OF STUDY

The present study included 126 persons selected largely, as in 1944, from groups subject to the physiologic stress of growth or recent pregnancy, since these conditions increase nutritional requirements. The subjects were distributed as follows: (1) 36 women who had been pregnant within the previous two years; (2) 32 children aged three to 6 years; (3) 21 children aged 10 to 14 years; (4) 11 men; (5) 16 other women; and (6) 10 children aged 7 to 9 years. The first 4 groups, numbering 100 persons in all, are comparable in size and composition to the 4 similar categories which comprised the 113 persons examined in 1944. Groups 5 and 6 were not represented in the earlier study.

The survey included: (1) a detailed dietary history; (2) a medical history and physical examination, with particular emphasis on findings indicative of nutritional disease; and (3) laboratory tests, including determinations of hemoglobin and protein (Phillips et al., '45), vitamin A and carotene (Bessey et al., '46), riboflavin (Burch et al., '48), and ascorbic acid (Lowry et al., '45). In 34 adult subjects, the urinary excretion of riboflavin (*L. casei*), thiamine (thiochrome with decalso absorption), and N′-methylnicotinamide (fluorometrically) was measured during 4 hours after administering an oral test dose of thiamine 5 mg, riboflavin 5 mg, and niacinamide 50 mg.

DIETARY STUDIES

Dietary observations included a history for each individual of all food ingested during the previous 24 hours, a record for each family unit of the frequency with which certain groups of foods were served, a history of the home production of food and an estimate of food purchases. The individuals studied represented 45 families.

The intake records were calculated for the usual nutrients (Boyd, Eads and Sandstead, '47, supplemented by Bowes and Church, '44). Values for fat salt beef were derived from those for cooked beef on the basis of 75% fat; those for moose were taken as being equivalent to available values for venison. Due to the custom of prolonged boiling of vegetables, the ascorbic acid content of these foods was calculated at 50% of table values. Recipes were obtained for all foods containing more than one ingredient (bread, cakes, pies, soups, stews, and so forth) and nutrients calculated from the values for each component.

Local food production is limited to small gardens (potatoes, turnips, cabbage, carrots, beets), eggs, fish, and a little milk. Table 1 indicates the number of families with some home production of foodstuffs. Three of the families produced none of their food supply. A few families had exhausted their store of potatoes in July. Root vegetables and cabbage are stored for use in the winter. Only 4 of the families had lettuce or used greens from their gardens. The home supply of fruits is preserved entirely as jam. Seven families had home-produced fresh milk at least 9 months of the year and 7 others bought one pint to one quart of fresh milk daily. Milk goats were kept by one family. Approximately 50% of the families in the study caught their own fish supply — usually cod, herring, and caplin caught in the winter, then dried or salted or both and stored. Lobster, salmon, and trout are caught in the spring and summer, some being canned for use later in the year. Thirty families kept hens and had eggs available; 5 ate chicken a few times in the spring. The only lean meat obtainable is wild game killed in the winter months and bottled for use during the rest of the year. Although 8 families kept sheep, these are seldom used for food.

The frequency of consumption of foods by 45 families (table 2) is indicative of the usual pattern of diet. As noted in the previous study (Metcoff, Goldsmith, McQueeney, Dove and Stare, '45), children from the time of weaning receive

the same food as adults. The basis of the diet consists of bread,[3] crackers, cereal grains, potatoes, desserts (cake, pie, cookies), molasses, jams, and tea (colored with one to two teaspoonfuls of evaporated milk). These foods are eaten two or more times a day. Vegetables are limited to those produced at home, with the exception of cabbage and turnips, which can be purchased during the winter months. Six families gave histories of having leafy green vegetables or carrots 4 times a week or more, in the summer, while 12 had

TABLE 1

Home food production by 45 families in Norris Point

	NO. OF FAMILIES
Gardens	
Potato only	4
Potato, cabbage, turnip, carrot, beet	38
Fruit (plum, raspberry, rhubarb)	4
Wild berries for jam	45
Milk cows (one per family)	7
Fish (cod, herring, caplin, lobster, trout)	22
Hens (from 5 to 30 per family)	30
Pigs	1
Cattle	3
Goats	1
Wild game (moose, rabbit, caribou)	37
Sheep	8

such foods only rarely or not at all. Over 50% of the families consume other vegetables (cabbage and turnips, mainly) as often as twice a week during all seasons of the year.

Fresh citrus fruits of inferior quality may be obtained in the spring, summer, and fall; canned citrus juices during the whole year. Ten families used citrus fruits 4 times a week or oftener, while 22 rarely or never had such foods. Tomatoes, usually canned, were used once a week or oftener by 30 families. They are usually added in small amounts as ingredients of soups or stews. Other fruits used consisted

[3] Homemade from enriched flour, yeast, and water.

mainly of apples and berries. Canned fruits are unavailable to most families because of the expense. Dried fruits were used at least once a week in small amounts by approximately 50% of the families as ingredients in cakes and cookies.

Of the protein foods, fish is consumed at least once a week or more often by 43 of the 45 families in the study. Small

TABLE 2

Frequency of consumption of foods by 45 families[1]

FOODS	DAILY	FOUR TIMES A WEEK	ONE TO THREE TIMES A WEEK	ONCE A MONTH OR LESS	NEVER
Bread, enriched	45	..	..	..	..
Crackers	35	3	4	..	3
Cereals, whole grain or enr.	16	8	14	3	4
non-enriched	..	1	19	22	3
Desserts	38	4	3	..	..
Fats					
butter	5	..	..	..	40
margarine	40	..	..	..	5
bacon	2	2	14	7	20
fat meat	4	3	20	11	7
Potatoes	45	..	..	..	..
Jam and jelly	36	7	1	1	..
Molasses	33	4	3	3	2
Tea	45	..	..	..	..
Fish	..	3	40	2	..
Eggs	9	7	21	3	5
Lean meat, incl. game meat	..	5	34	6	..
Cheese	..	1	13	13	18
Dried peas and beans	..	3	37	3	2
Nuts, peanut butter	2	2	5	9	27
Dried fruits	1	1	19	15	9
Citrus fruits or juices	2	8	13	13	9
Other fruits, raw or canned	1	1	21	12	10
Tomatoes	..	6	24	11	4
Vegetables, leafy gr. and yellow[2]	3	3	27	5	7
Vegetables, other	3	5	25	9	3
Ice cream[2]	6	2	18	10	9
Soft drinks[2]	6	5	8	5	21

[1] Milk was consumed frequently in very small amounts. The quantities were so small that frequency is a poor indication of the consumption. For estimates of amounts see table 3.

[2] Summer only.

amounts of lean meat (mainly wild game and a little canned beef) are included once a week or oftener in the diet of 39 of the families. For 90% of the families studied, dried beans constitute as important a source of protein as fish — large servings being included in the diet at least once a week. The consumption of eggs depends largely on their production at home, as few can be procured on the market. The statement that 37 families ate eggs oftener than once a week holds for only some 6 months of the year.

As has been noted, local milk production is limited. Imported evaporated milk is used in the diet (for all except babies) chiefly as an adjunct to hot tea.[4] It is never used in

TABLE 3

Grams of milk consumed per day (calculated on whole milk basis)

QUARTILES	WOMEN		MEN	CHILDREN		
	Post partum	Other		3–6 years	7–9 years	10–14 years
First	48	70	0	60	40	40
Second	116	150	90	120	62	330
Third	241	306	770	378	150	630

cooking and only rarely in cocoa, when it is diluted about 5 times with water. To determine the actual amount of fluid milk consumed by the persons in the study, the 24-hour diet histories were checked and the total milk consumption was calculated on the basis of grams of whole fluid milk. Table 3 presents the quartiles and medians (second quartiles) in grams of whole milk consumed per day.

All groups showed a deficiency in milk consumption which was pronounced for the post partum women and for children aged three to 6 and 7 to 9 years. Four women in the post partum group drank no milk, and two individuals in each of the other 5 age and sex groups gave a negative history for milk consumption in the 24-hour period studied.

[4] During school months, children receive approximately 180 ml per day of a milk-cocoa drink. See footnote 2, page 43.

Data for the daily intakes of various food constituents by quartiles for post partum women and children are given in table 4 in comparison with the results of several other surveys and with the National Research Council recommended allowances.

The intake of calories, protein, thiamine, iron, and niacin in Norris Point was similar to that reported in other areas and approximates adequacy. The intake of riboflavin and calcium was much lower in Norris Point than elsewhere. The first and second quartiles for vitamin C, and to a lesser extent for vitamin A, were considerably lower than those recorded in the other surveys.

Comparison of the findings in Norris Point with the recommended dietary allowances of the Food and Nutrition Board ('48) shows that of the post partum women, 75% received less calcium and riboflavin and 50% fewer calories and less protein, iron, vitamin A, thiamine, and ascorbic acid than are suggested. Of the children aged three to 6, 75% received less than the recommended allowance for calcium and 50% failed to meet the standards for protein, vitamin A, riboflavin, and ascorbic acid. Of the children aged 10 to 14, 75% failed to attain the standards for calcium and ascorbic acid, while 50% were below them in calories, protein, vitamin A, and riboflavin.

In all groups the first quartiles were very low in calcium and ascorbic acid and quite low in vitamin A. Less than liberal were the intakes of riboflavin and, in some groups, of protein.

Clinical findings

A medical history was taken of all individuals over 10 years of age. The incidence of symptoms which may occur in deficiency states was remarkably similar to that in the 1944 findings (table 5). We feel that little significance should be attached to symptoms alone as a measure of nutritional status. One might be tempted to interpret the slight decrease (not statistically significant) in gastrointestinal complaints

TABLE 4

Calculated daily intake of nutrients in Norris Point as compared to results of similar studies elsewhere

SURVEY	CHILDREN 3–6 YRS.				CHILDREN 10–14 YRS.				POST PARTUM WOMEN			
	1st quart.	Median	3rd quart.	Mean	1st quart.	Median	3rd quart.	Mean	1st quart.	Median	3rd quart.	Mean
Calories												
Norris Point	1224	1643	2113	1735	1613	2199	2640	2263	1302	1922	2657	2089
Virginia (spring)[1]	1388	1599	2008	1720	....	....	....	....	1346	1678	1975	1676
Virginia (fall)[1]	1619	1811	2242	1939	....	....	....	....	1492	1715	2009	1809
Tenn., Williamson County[2]	1557	1850	2362	1933	1864	2230	2591	2266	1688	2250	2958	2346
Tenn., Wilson County[3]	1297	1484	1833	1547	1422	1679	2225	1855	1346	1678	2048	1736
N.R.C. allowances[4]	....	....	....	1600	....	....	....	2500	....	....	....	2500
Protein (gm)												
Norris point	30	38	55	46	45	65	81	66	44	54	77	62
Virginia (spring)[1]	50	58	71	60	..	..	..	..	47	60	71	60
Virginia (fall)[1]	52	60	68	60	..	..	..	..	44	56	67	56
Tenn., Williamson County[2]	50	62	75	63	56	75	87	71	54	72	89	72
Tenn., Wilson County[3]	35	48	60	47	40	54	73	57	39	53	65	56
N.R.C. allowances[4]	..	..	..	50	..	..	..	70	..	..	..	85
Fat (gm)												
Norris Point	36	48	87	66	46	67	110	79	47	64	102	78
Tenn., Wilson County[3]	51	60	73	62	52	66	92	72	50	64	79	67
Vitamin C (mg)												
Norris Point	4	13	50	31	12	20	63	39	8	26	79	58
Virginia (spring)[1]	26	36	43	38	..	..	..	..	22	44	63	45
Virginia (fall)[1]	22	32	43	34	..	..	..	..	18	32	44	36
Tenn., Williamson County[2]	22	34	53	38	27	40	55	41	28	40	54	38
N.R.C. allowances[4]	..	..	..	50	..	..	..	75	..	..	..	100
Total vitamin A (I.U.)												
Norris Point	1378	2107	4584	2965	1620	3616	5943	5344	1851	2959	5486	6018
Virginia (spring)[1]	2810	3984	5666	4730	....	....	....	....	2939	4942	8828	6318
Virginia (fall)[1]	2742	3875	4845	4068	....	....	....	....	3148	4524	6495	5186
Tenn., Williamson County[2]	2188	3600	4875	3878	2283	3312	6250	4125	2625	5750	7688	5133
Tenn., Wilson County[3]	2264	3150	4650	3712	2650	3692	5781	4254	2875	4913	9688	
N.R.C. allowances[4]	....	....	....	2500	....	....	....	4500	....	....	....	6000
Thiamine (mg)												
Norris Point	.46	.64	.90	.72	.62	.85	1.08	.89	.52	.76	1.05	.83
Virginia (spring)[1]	.58	.70	.85	.73	....	....	....	....	.56	.72	.84	.72
Virginia (fall)[1]	.66	.77	.92	.79	....	....	....	....	.63	.73	.87	.76
Tenn., Williamson County[2]	.61	.77	.97	.80	.74	.94	1.14	.96	.70	.96	1.23	.98
Tenn., Wilson County[3]	.54	.66	.80	.68	.59	.77	.96	.80	.56	.77	.98	.81
N.R.C. allowances[4]	....	....	....	.7	....	....	....	1.2	....	....	....	1.2
Riboflavin (mg)												
Norris Point	.61	.85	1.19	.94	.82	1.12	1.43	1.17	.69	1.00	1.39	1.09
Virginia (spring)[1]	.77	.93	1.13	.96	....	....	....	....	.74	.95	1.11	.95
Virginia (fall)[1]	.87	1.02	1.22	1.04	....	....	....	....	.83	.97	1.15	1.00
Tenn., Williamson County[2]	.80	1.02	1.28	1.06	.98	1.24	1.51	1.27	.92	1.27	1.63	1.30
Tenn., Wilson County[3]	.71	.87	1.06	.90	.78	1.02	1.27	1.06	.74	1.02	1.30	1.07
N.R.C. allowances[4]	....	....	....	1.2	....	....	....	1.7	....	....	....	1.7
Niacin (mg)												
Norris Point	5.4	7.5	10.5	8.3	7.2	9.9	12.6	10.3	6.1	8.8	12.3	9.6
Virginia (spring)[1]	6.8	8.2	10.0	8.5	....	....	....	....	6.5	8.4	9.8	8.4
Virginia (fall)[1]	7.7	9.0	10.8	9.2	....	....	....	....	7.3	8.6	10.1	8.8
Tenn., Williamson County[2]	7.1	9.0	11.3	9.3	8.6	10.9	13.3	11.2	8.1	11.2	14.4	11.4
Tenn., Wilson County[3]	6.3	7.7	9.4	7.9	6.9	9.0	11.2	9.3	6.5	9.0	11.5	9.4
N.R.C. allowances[4]	....	....	....	12	....	....	....	15	....	....	....	15

Thiamine (mg)												
Norris Point	0.66	1.06	1.19	0.98	1.10	1.27	1.60	1.37	0.82	1.09	1.41	1.17
Virginia (spring)[1]	0.79	0.93	1.09	0.97	...	...	...	...	0.74	0.94	1.20	0.98
Virginia (fall)[1]	0.76	0.88	1.11	0.93	...	...	...	...	0.71	0.86	1.03	0.99
Tenn., Williamson County[2]	0.90	1.23	1.52	1.21	1.01	1.32	1.85	1.46	0.80	1.05	1.75	1.32
N.R.C. allowances[4]	...	...	...	0.80	...	...	...	1.20	...	...	...	1.80
Riboflavin (mg)												
Norris Point	0.68	0.90	1.50	1.15	1.00	1.54	2.00	1.62	0.75	1.04	1.58	1.33
Virginia (spring)[1]	1.84	2.29	2.60	2.31	...	...	...	...	1.68	2.12	2.77	2.14
Virginia (fall)[1]	1.84	2.29	2.58	2.30	...	...	...	...	1.46	2.00	2.34	1.97
Tenn., Williamson County[2]	1.39	1.85	2.41	1.81	1.56	1.90	2.51	1.98	1.16	1.50	2.18	1.71
N.R.C. allowances[4]	...	...	...	1.2	...	...	...	1.8	...	...	...	2.5
Niacin (mg)												
Norris Point	5.9	8.1	11.9	9.1	9.0	14.6	17.4	14.0	8.9	12.3	16.9	13.2
Virginia (spring)[1]	5.0	6.0	8.0	7.0	..	....	....	....	5.0	7.0	8.0	7.0
Virginia (fall)[1]	6.0	8.0	9.0	8.0	..	...	...	...	6.0	7.0	9.0	8.0
Tenn., Williamson County[2]	6.2	8.0	10.1	8.1	8.1	9.6	12.2	9.7	6.9	8.0	12.5	9.5
N.R.C. allowances[4]	..	..	..	8	..	...	..	12	..	..	...	18
Calcium (mg)												
Norris Point	227	402	680	556	356	812	976	776	256	419	671	554
Virginia (spring)[1]	890	1166	1368	1143	...	...	...	...	737	1092	1329	1032
Virginia (fall)[1]	856	1109	1324	1141	...	...	...	...	796	975	1144	960
Tenn., Williamson County[2]	640	850	1110	880	660	900	1230	960	520	950	1180	910
Tenn., Wilson County[3]	368	675	....	...	379	575	950	...	337	543	831	...
N.R.C. allowances[4]	...	...	....	1000	...	...	...	1200	...	...	...	1500
Iron (mg)												
Norris Point	6.1	9.5	12.8	10.1	11.4	14.2	16.6	15.3	8.4	11.4	15.8	12.9
Virginia (spring)[1]	7.0	8.0	11.0	9.0	...	...	...	...	8.0	10.0	12.0	10.0
Virginia (fall)[1]	8.0	10.0	12.0	10.0	...	...	...	...	8.0	10.0	12.0	10.0
Tenn., Williamson County[2]	8.4	11.3	13.0	10.9	10.3	12.1	15.1	12.7	9.2	14.0	16.4	12.9
N.R.C. allowances[4]	..	...	...	8.0	...	...	...	12.0	..	...	...	15.0

[1] Dean, '49.
[2] Robinson, Steinkamp and Kaser, '44.
[3] Youmans et al., '43a, b, '44a, b.
[4] Food and Nutrition Board, '48.

TABLE 5

Comparative incidence of symptoms possibly related to nutritional deficiency in Norris Point in 1944 and 1948

	1944	1948
Number of histories obtained	79	68
	PER CENT WITH SYMPTOMS	
Skin:		
Easy bruising	23	24
Dermatitis on exposed surfaces	0	0
Eyes:		
Burning or itching	35	35
Photophobia	30	24
Lacrimation	32	28
Visual fatigue with blurring	11	31
Night blindness	10	7
Snow blindness	16	10
Mouth:		
Burning, painful tongue	22	19
Cracked, painful lips	27	28
Sores at angles of mouth	4	25
Bleeding gums	37	37
Cardiorespiratory:		
Exertional dyspnea	22	21
Palpitation	19	19
Precordial pain	4	6
Edema (dependent)	1	6
Gastrointestinal:		
Anorexia	11	9
Indigestion	27	18
Chronic diarrhea	4	1
Hemorrhoids with bleeding	16	12
Neuromuscular-skeletal:		
Numbness and tingling	24	18
Syncope or dizziness	18	21
Burning feet	6	13
Leg cramps	23	25
General:		
Nervousness and irritability	23	13
Easy fatigability	15	16

as a reflection of increased thiamine intake, inasmuch as so-called "Newfoundland stomach" (frequently observed digestive disturbances) has, in the past, been attributed by some to a deficiency of this B vitamin. Caution must be enjoined in any such interpretation, however, as one cannot be certain that a real decrease occurred.

The physical examinations in 1948 showed a number of definite differences from those of 1944. In the earlier survey both adults and children appeared apathetic and lacking in spontaneity and initiative. The children exhibited little curiosity, appeared older than their years, were inactive and quiet, and seldom engaged in spontaneous play. By contrast, in 1948 the apathy had largely disappeared; more initiative, curiosity, and interest were apparent; the children seemed more independent, played spontaneously and showed considerable inquisitiveness. It has been remarked (Metcoff et al., '45) that the mental and emotional reactions noted in 1944 were similar to those reported in experimentally induced vitamin B complex deficiency. While many factors, including acquaintance with and acceptance of the survey team, were unquestionably operative in producing the changes noted in 1948, it is conceivable that an increase in the intake of the B vitamins may have contributed to these changes.

The incidence of clinical signs which may be related to malnutrition is given in table 6 for the two surveys. The criteria for judging abnormality were essentially the same in 1948 as in 1944, although a few additional physical signs were recorded in the second study. The incidence of the findings is indicated separately for each population group, as well as for the entire sample, in the 1948 survey, while only total incidence is given for 1944. Age-sex separations of the earlier data are given in the initial report (Metcoff et al., '45).

There were slight differences in the composition of the total sample in the two surveys. The incidence of abnormal

TABLE 6

Percentage incidence of physical signs suggesting nutritional deficiency in Norris Point in 1948 and 1944

	1948 SURVEY							1944 SURVEY
	Women		Children			Men	Total	Total
GROUP	Recently pregnant	Other	3–6 yrs.	7–9 yrs.	10–14 yrs.			
Number of persons examined	36	16	32	10	21	11	126	113
	%	%	%	%	%	%	%	%
Signs of abnormality								
Hair — dry, "staring"	0	0	16	20	5	0	6	..
Skin facial								
Seborrhea, nasolabial	3	6	3	10	0	0	3 [1]	18
Seborrhea, other	6	0	37	50	14	0	17	10
Erythema	3	0	31	40	19	9	16 [1]	7
Folliculosis	6	6	0	0	10	0	4	..
Skin general								
Follicular keratosis	50	37	44	60	48	0	43	44
Dryness and scaling	11	6	3	30	5	0	8	10
Perifolliculosis	36	12	0	10	0	0	13	..
Acneiform eruption	6	6	0	0	0	0	2	4
Thick pigmented pressure points	19	25	0	10	14	0	12	20
Purpura and petechiae	8	6	0	0	0	0	3	4
Bluish-red extremities	44	31	22	40	52	0	34 [1]	18
Pellagraform lesions	0	0	0	10	0	0	1	0
Eyes								
Thickened conjunctiva	97	75	3	40	38	82	55	65
Circumcorneal injection	17	19	0	30	10	9	12 [1]	25
Conjunctival injection	31	56	22	40	38	27	33	39
Blepharitis	11	6	3	10	0	36	9	..
Lips and mouth								
Angular lesions	8	19	12	20	14	18	13	21
Angular scars	25	31	6	30	5	0	16	..
Cheilosis	31	56	22	40	38	27	33	..

Conjunctival injection	31	25	25	10	0	36	9	..
Blepharitis	11	6	3	10	0	36	9	..
Lips and mouth								
Angular lesions	8	19	12	20	14	18	13	21
Angular scars	25	31	6	30	5	0	16	..
Cheilosis	31	25	37	50	48	0	33 [1]	6
Pallor	0	0	0	0	0	0	0	9
Ulcers of mouth	0	0	0	0	0	0	0	3
Tongue								
Atrophy or hypertrophy of papillae — Total	50	56	34	80	57	0	46 [1]	77
Slight	28	37	22	70	33	0	29	39
Moderate	22	12	12	10	24	0	16	19
Marked	0	6	0	0	0	0	1 [1]	19
Purple color	6	0	6	0	0	0	3 [1]	30
Red tip and/or sides	19	31	12	30	14	0	17	18
Fissures	25	25	0	0	19	0	13	19
Erosion or ulcers	0	0	0	0	0	0	0	4
Serrations — swelling	6	0	0	0	0	0	2	3
Teeth								
Caries (total)	81	62	75	90	76	73	76	84
Edentulous	14	37	0	0	0	27	11	6
Gums (all changes)	72	62	19	50	67	64	54	50
Marginal redness	58	50	3	30	38	36	36	..
Marginal swelling	64	56	19	50	57	18	45	..
Atrophy of papillae	31	31	0	0	0	18	14	..
Recession	50	44	3	10	14	45	28	..
Bleeding gums	33	12	0	0	0	0	11	14
Neuromuscular								
Calf tenderness	3	6	0	0	0	0	2	7
Loss of ankle jerk and knee jerk	6	12	0	0	0	0	3	4
Plantar dysesthesia	0	0	0	0	0	0	0	4
Motor weakness	0	0	0	0	0	0	0	1
Vibratory sensation lost	0	0	0	0	0	0	0	4
Cardiovascular								
Systolic murmur	22	31	3	10	0	9	13 [1]	34

[1] Significant difference between 1944 and 1948 $P = < 0.01$.

physical findings in 1948 was essentially the same whether the entire sample or only the groups comparable to 1944 were compared.

Comparison of the findings in 1948 with those of 1944 shows a statistically significant [5] decrease in the incidence of nasolabial seborrhea, circumcorneal injection, atrophy and hypertrophy of the lingual papillae, purple or magenta tongue, and systolic heart murmurs. The decrease in the incidence and severity of glossitis was remarkable. Pronounced abnormality of the lingual papillae was noted in only one person in 1948, while 19% of the population showed advanced changes in 1944. The frequency with which magenta tongue was observed decreased from 30 to 3%. A decrease in the incidence of neurological abnormalities was also noted in 1948. As so few subjects exhibited signs related to the nervous system in 1944, the meaning of this decrease cannot be determined.

A statistically significant increase in the incidence of facial erythema and seborrhea (other than nasolabial), of bluish-red mottling of the extremities, and of cheilosis was observed in 1948. Greater attention in the second survey to the recording of changes in skin color may account for the increase in this area, but not for the greater frequency of seborrhea or cheilosis, which were searched for with equal thoroughness on both occasions.

The incidence of the other clinical findings listed in table 6 was not significantly different in the two surveys. A large percentage of the persons examined had follicular keratosis, thickened conjunctivae, dental caries, and abnormalities of the gums. In children aged three to 6 years, but not in other age groups, significantly less thickening of the conjunctivae was observed in 1948 than in 1944. It may be pointed out that the frequency of angular stomatitis and of thickening and pigmentation of the elbows and knees was diminished in 1948, but not to a level of statistical significance.

[5] Significant at a level of one in 100, i.e., $P < 0.01$.

Comparison of findings in the various groups, omitting adult males of which only 11 were examined, shows several interesting features. The incidence of thickened conjunctivae and gum changes increased with age. Conjunctival and circumcorneal injection and atrophy or hypertrophy of the lingual papillae were slightly less frequent in children aged three to 6 than in other groups, while fissuring of the tongue was present only in persons over the age of 14. Follicular keratosis, angular stomatitis, cheilosis and dental caries were observed with equal frequency in all groups. The incidence of perifolliculosis was high only in recently pregnant women; purpura and petechiae were noted only in adult females. Seborrheic lesions were more common in children than in adults, while dry "staring" hair was observed solely in the younger age groups.

The incidence of abnormal physical signs in recently pregnant adult women differed little from that found in other women. The prevalence of follicular keratosis, xerosis, perifolliculosis, and gum changes was slightly higher, that of angular stomatitis lower, in the recently pregnant group.

The height and weight of all persons in the survey were measured without shoes and with sweaters or coats removed. Figures 1 and 2 compare the findings in children in both surveys to data for healthy white American children in Massachusetts and Iowa (Vickers and Stuart, '43; Meredith, '35; Boynton, '36). In 1944, 15 of 29 boys were of shorter stature than 80% of the American boys, while in 1948 only 5 of 26 boys were below the 80% range. A similar trend toward increased height was noted for the girls between 1944 and 1948. The weight of the Newfoundland children aged three to 6 was essentially within the normal range in both 1944 and 1948. The weight of the older children was below the American standards in both surveys, thinness being perhaps a little more noteworthy in 1948.

It was noted in 1944 that the adults studied were generally of short stature and thin. Only one of 39 women was definitely obese. In 1948, 11 of 52 women examined were more than

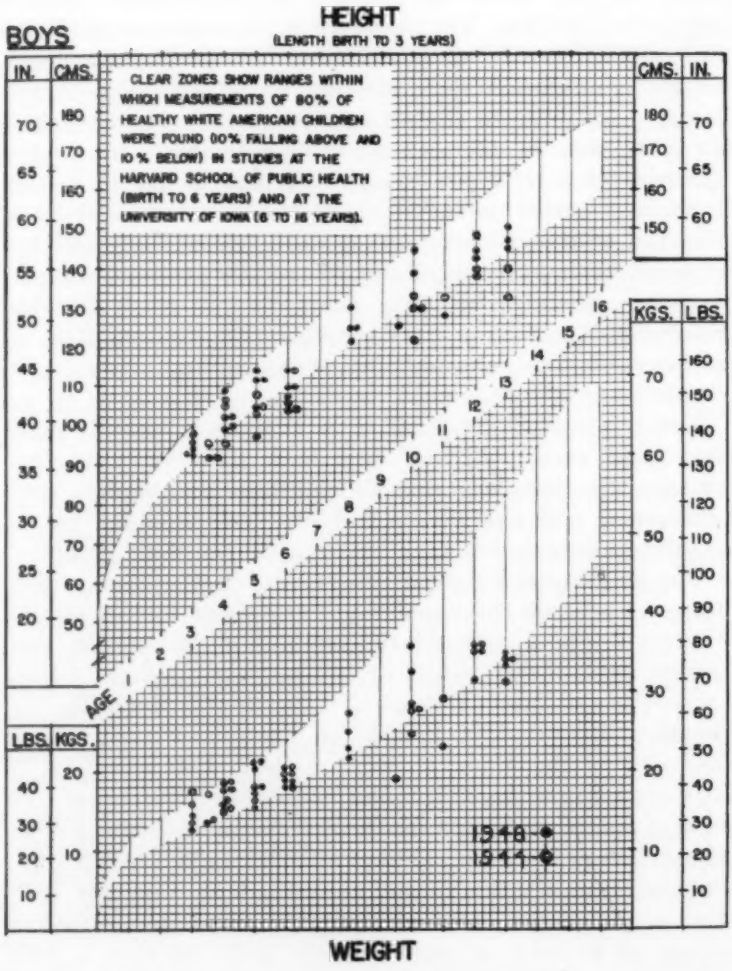
BOYS
HEIGHT
(LENGTH BIRTH TO 3 YEARS)
CLEAR ZONES SHOW RANGES WITHIN WHICH MEASUREMENTS OF 80% OF HEALTHY WHITE AMERICAN CHILDREN WERE FOUND (10% FALLING ABOVE AND 10% BELOW) IN STUDIES AT THE HARVARD SCHOOL OF PUBLIC HEALTH (BIRTH TO 6 YEARS) AND AT THE UNIVERSITY OF IOWA (6 TO 16 YEARS).
IN.
CMS.
KGS.
LBS.
AGE
1948
1944
WEIGHT

Fig. 1 Height and weight of Newfoundland boys compared with American boys.

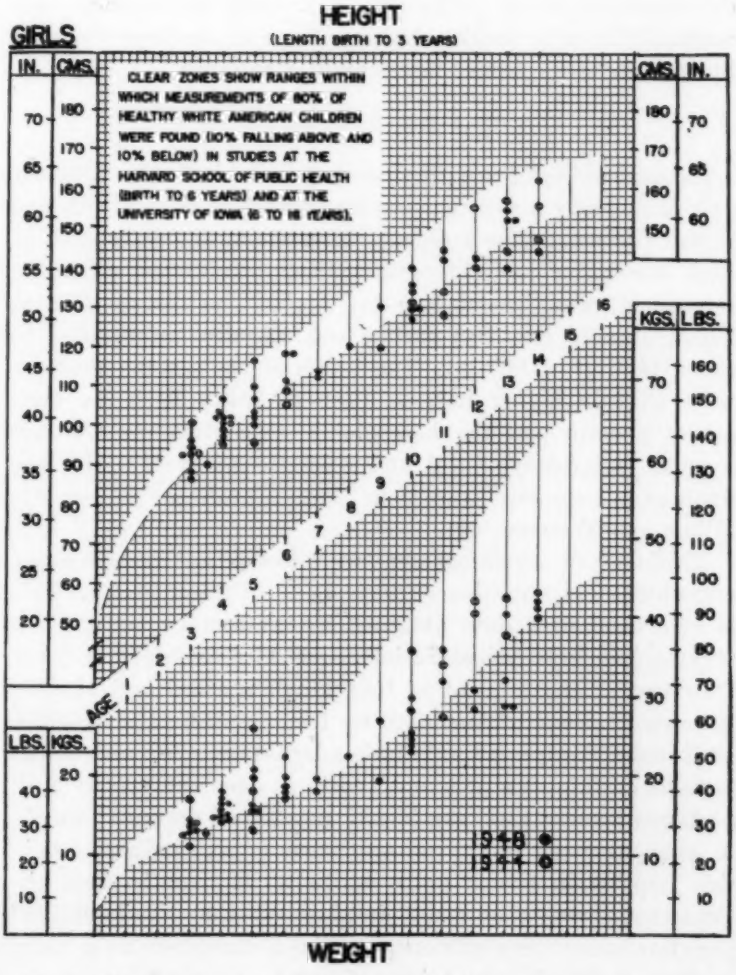
HEIGHT
(LENGTH BIRTH TO 3 YEARS)
GIRLS
IN.
CMS.
CLEAR ZONES SHOW RANGES WITHIN WHICH MEASUREMENTS OF 80% OF HEALTHY WHITE AMERICAN CHILDREN WERE FOUND (10% FALLING ABOVE AND 10% BELOW) IN STUDIES AT THE HARVARD SCHOOL OF PUBLIC HEALTH (BIRTH TO 6 YEARS) AND AT THE UNIVERSITY OF IOWA (6 TO 18 YEARS).
KGS.
LBS.
AGE
1948
1944
WEIGHT

Fig. 2 Height and weight of Newfoundland girls compared with American girls.

20% overweight, according to the tables of standard weight developed by Wood (Bigwood, '39). Only two women were underweight to a similar degree. None of the men deviated by 20% or more from these weight standards.

Laboratory findings

Laboratory data are summarized in table 7. In 1944 the only laboratory measurements made were of hemoglobin and serum proteins. The incidence of anemia was judged to be high according to arbitrarily chosen standards which, in retrospect, were themselves too high. The distribution of hemoglobin in the 1948 survey was slightly higher than in the 1944 study (table 8). The levels of the plasma proteins were slightly higher in 1948 than in 1944. Both the total serum protein and the hemoglobin concentrations were at a quite satisfactory level and compare favorably with the findings in extensive studies in North Carolina (Milam, '46; Milam and Muench, '46).

The levels of serum carotene were lower than those usually encountered in nutrition surveys in the United States. This is in accord with data obtained in the dietary study. The serum vitamin A concentrations were at a satisfactory level. Only two of the total of 84 individuals studied had vitamin A concentrations of less than 60 I.U. per 100 ml. Inasmuch as decreased capacity for dark adaptation, the earliest detectable certain sign of avitaminosis A, does not appear until a plasma level of less than 50 I.U. per 100 ml occurs (Vitamin A Subcommittee, '49), the existence of avitaminosis A in this population seems quite unlikely. It may be that the somewhat lower-than-usual intakes of the Newfoundland population are very efficiently utilized, inasmuch as a large portion of the vitamin A is preformed.

While the mean serum ascorbic acid levels were not indicative of saturation of the several groups in the population, their distribution was such that one would not anticipate the

TABLE 7

Chemical findings in the blood in Norris Point survey, 1948

TOTAL SERUM PROTEIN (gm per 100 ml)				
	CHILDREN		WOMEN [1]	TOTAL
	3–6 yrs.	10–14 yrs.		
NO. PERSONS	28	21	36	85
1st quartile	6.3	6.7	7.0	6.7
Median	6.7	7.0	7.4	7.0
3rd quartile	7.0	7.8	7.8	7.4
Mean	6.7	7.2	7.2	7.0

SERUM CAROTENE (μg per 100 ml)				
NO. PERSONS	21	19	36	76
1st quartile	14	42	34	29
Median	36	59	54	53
3rd quartile	64	92	84	85
Mean	47	70	63	60

SERUM VITAMIN C (mg per 100 ml)				
NO. PERSONS	27	21	36	84
1st quartile	0.15	0.16	0.08	0.13
Median	0.24	0.56	0.34	0.39
3rd quartile	0.64	0.84	0.57	0.65
Mean	0.49	0.70	0.41	0.51

HEMOGLOBIN (gm per 100 ml)			
CHILDREN		WOMEN [1]	TOTAL
3–6 yrs.	10–14 yrs.		
28	21	36	85
11.6	11.7	11.9	11.6
11.8	12.2	12.6	12.2
12.2	12.8	13.4	12.9
12.0	12.5	12.6	12.3

SERUM VITAMIN A (I.U. per 100 ml)			
21	19	36	76
79	79	109	92
104	104	123	119
149	131	158	148
120	106	135	124

SERUM RIBOFLAVIN VALUES (μg per 100 ml) (34 women)[1]

Free riboflavin	Bound riboflavin	Total riboflavin
0.85	1.72	2.60
1.14	2.05	3.07
1.49	2.62	3.92
1.22	2.15	3.38

[1] Pregnancy within previous two years.

TABLE 8

Comparison of hemoglobin distributions in 1944 and 1948 [1]

WOMEN			CHILDREN 3–6 YEARS			CHILDREN 10–14 YEARS		
Year	1944	1948		1944	1948		1944	1948
Number of persons	37	36		21	28		21	21
Hemoglobin gm %	% Cases	% Cases	Hemoglobin gm %	% Cases	% Cases	Hemoglobin gm %	% Cases	% Cases
< 10.0	3	3	< 9.5	0	0	< 10.0	0	0
10.0–11.5	30	17	9.6–10.5	5	0	10.1–11.0	9.5	0
11.6–12.5	27	22	10.6–11.5	35	21	11.1–12.0	9.5	29
> 12.5	40	58	> 11.5	60	79	> 12.0	81.0	71

[1] This distribution is on the basis of standards used in judging anemia in 1944. It is now felt that these standards were too high but they are unchanged to permit comparison of data.

occurrence of clinical scurvy in this population. The medical studies confirm this position.

An examination of the distributions by quartiles of the values for serum carotene, ascorbic acid, and, to a lesser extent, serum riboflavin demonstrates a point worthy of note. There is definite skewness of these distributions, as is revealed by the fact that the median in each instance is somewhat lower than the mean. The third quartile in each case is at a very satisfactory level. At the other extreme, the first quartile in each group is at a somewhat unsatisfactory concentration. Similar distributions exist in the dietary intakes. Does this not mean that the most effective measures for nutritional improvement will be those that will favorably alter the nutriture of the individuals falling into the lower quartile? It is apparent that there exists within the population here studied a definite percentage of well-nourished individuals; and, accordingly, additional nutritional improvement does not necessarily have to be population-wide. In future nutrition studies it would seem important to define the characteristics (economically and sociologically) of this most poorly nourished segment, in order to plan effective remedial measures.

The urinary excretion of thiamine, riboflavin, and N′-methylnicotinamide was measured in 34 adults for the 4-hour period immediately following an oral dose of 5 mg each of thiamine and riboflavin, and 50 mg of nicotinamide. The results of the load tests for these three B vitamins are presented in table 9 and are compared with data on 17 healthy subjects in New Orleans. The mean and range of excretion of thiamine and N′-methylnicotinamide were essentially the same in the Newfoundland as in the New Orleans subjects. Only one person in each study had a thiamine output of less than 100 μg. No subjects had a low excretion of N′-methylnicotinamide. The excretion of riboflavin was considerably lower in the Newfoundland than in the New Orleans group, and in 9 of 34 Newfoundland subjects the output was less than 1.2 μg — a level which has been tentatively considered

the lower limit of normal. Mean values for both free and total riboflavin in the serum [6] in these 34 adults were essentially the same as those found by Bessey in normal persons. In one-fourth of the Newfoundland subjects, total riboflavin in the serum was less than 2.6 μg %, the lowest value reported by Bessey.

Attempts were made to determine whether any correlation existed among various physical signs generally considered to suggest a deficiency of certain nutrients, laboratory findings related to these factors, and the dietary intake of the nutrients in question. Correlation between the dietary intake

TABLE 9

Four-hour urinary excretion after an oral dose of 5 mg thiamine, 5 mg riboflavin, and 50 mg nicotinamide

PLACE AND NO. OF SUBJECTS	EXCRETION	THIAMINE	N′-METHYL-NICOTINAMIDE	RIBOFLAVIN
		μg	*mg*	*mg*
Norris Point, Newf.	Mean ± S.D.[1]	264 ± 113	8.2 ± 2.8	1.4 ± 0.3
(34)	Range	77–548	4.0–17.3	0.7–2.5
New Orleans	Mean ± S.D.	224 ± 84	6.8 ± 2.6	2.0 ± 0.6
(17)	Range	82–480	3.4–12.5	1.3–3.9

[1] S.D. = standard deviation.

of niacin and the excretion of N′-methylnicotinamide was + 0.39 (P = 0.01), while that between the dietary intake of protein and the excretion of N′-methylnicotinamide was + 0.36 (P = 0.02). These correlations, while low, appear to be significant. There were no other definite correlations of significance in regard to any of the nutrients studied.

DISCUSSION

The nutritional improvement observed in Norris Point in 1948, as compared to 1944, was most definite in relation to symptoms and signs commonly associated with vitamin B

[6] For these determinations we are indebted to Dr. Nevin S. Scrimshaw and Miss Ruth Goodland of the University of Rochester School of Medicine, Rochester, N. Y.

complex deficiency. The increase in alertness of the adults and in spontaneity and activity of the children may be due to an improved nutriture or may be dependent on other factors. The satisfactory level of excretion of thiamine in the urine, the adequacy of dietary intake of thiamine in most subjects, and the small incidence of neurologic abnormalities indicate that thiamine status was reasonably satisfactory in 1948. The pronounced decrease in glossitis, the almost complete disappearance of purplish discoloration of the tongue, and the lower incidence of nasolabial seborrheic lesions and circumcorneal injection all suggest improved riboflavin and niacin nutrition. Urinary excretion of niacin was in the normal range, but that of riboflavin was low in approximately one-fourth of the subjects examined. The dietary supply of riboflavin was also low in about 25% of the group. The increased incidence of cheilosis in the second study cannot be interpreted. The slight diminution in angular stomatitis, though not significant, is consistent with the lingual changes.

Whether the observed non-significant changes in hemoglobin should be related to iron intake cannot be decided.

In general, the observed changes in clinical findings discussed above are consistent with the increase of nutrient intake provided by enriched flour.

There appeared to be no improvement in ascorbic acid nutrition in 1948. Both the intake and serum level of this vitamin were low in a sizeable segment of the population. The incidence of lesions of the gums was unchanged between the two surveys. It should be pointed out that the significance of redness and swelling of the gums as evidence of ascorbic acid deficiency is still debatable.

No change was observed in the prevalence of follicular keratosis or xerosis of the skin, which are sometimes related to vitamin A deficiency. The laboratory studies do not indicate that these signs in this population are due to avitaminosis A. While levels of carotene in the blood were low in many subjects, those of vitamin A were essentially normal. These findings are in keeping with those of Aykroyd et al.

('49) in their simultaneous study in the eastern section of Newfoundland. The dietary intake of vitamin A was low in more than one-fourth of the subjects and that of carotene extremely low in a majority of instances. Whether the decrease in conjunctival thickening in children aged three to 6 was related to improved vitamin A nutrition is unknown. Whether the normal serum vitamin A levels observed in the 1948 survey can be attributed to the fortification of margarine cannot be determined, since the 1944 study failed to include these determinations. The low values observed by Adamson et al. ('45) would suggest that there may have existed similar low levels at Norris Point in 1944 and that an increase occurred.

The findings in this resurvey in Newfoundland are remarkably similar to those reported by Aykroyd et al. ('49), who conducted a resurvey in the eastern area of the country in 1948. These workers noted a diminution in the prevalence of lesions which may be related to thiamine, riboflavin, niacin, and vitamin A deficiency, and commented on the greater alertness of the people and the decrease in dyspepsia and constipation. No improvement was noted in lesions possibly related to ascorbic acid deficiency, namely, redness and swelling of the gums, while perifolliculosis was higher in 1948 than in 1944. A significantly greater per cent of their subjects in the out-ports had low ascorbic acid in the blood in 1948 than in the earlier survey, while no change had occurred in St. Johns. Significantly higher levels of vitamin A in the blood and an increase in the urinary excretion of thiamine and riboflavin were observed in 1948, corroborating clinical findings. A positive correlation was demonstrated between the urinary excretion of thiamine and that of riboflavin. This was interpreted as indicating that the increased supply of these vitamins was derived from the same source — which appeared to be enriched flour. Aykroyd et al., in summarizing their findings, stated that the signs and symptoms of malnutrition which could have been expected to decrease in prevalence due to fortification of margarine with vitamin

A and enrichment of flour with thiamine, riboflavin, and niacin, were less frequent and less severe in 1948 than in 1944. The prevalence of lesions which could not be affected by these measures remained unaltered or increased.

The present report differs from that of Aykroyd et al., chiefly in that the prevalence of some clinical signs which may be related to vitamin A or riboflavin deficiency was not diminished in 1948. However, the incidence of many signs related to vitamin B complex deficiency was decreased and both dietary and laboratory studies corroborated, in general, the clinical observations.

SUMMARY

A resurvey of a selected sample of the population of Norris Point, Newfoundland, in 1948 showed that considerable improvement had occurred in the general nutritive state since 1944. A striking decrease in the incidence of several signs and symptoms related to vitamin B complex deficiency was observed. This improvement appeared to be related, in large part, to the use of enriched flour beginning in 1944. No improvement was noted in ascorbic acid nutrition and few changes occurred relative to vitamin A.

Newfoundland is a particularly favorable location for evaluating measures designed to alleviate malnutrition. Since much remains to be done to bring the nutrition of the population of Newfoundland up to the standard level of neighboring countries, future studies in this area would appear desirable.

ACKNOWLEDGMENTS

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THE INFLUENCE OF TRYPTOPHAN UPON URINARY NITROGEN AND AMINO ACID EXCRETION IN THE RAT [1]

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TWO FIGURES

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Investigation by Brush, Willman and Swanson ('47) on the role of amino acids and choline in nitrogen metabolism in the adult, male, albino rat led to the conclusion that cystine, choline and all the essential amino acids except phenylalanine, valine, and tryptophan, exert some action in sparing body nitrogen. Their studies on the effects of egg protein and of a mixture of the essential amino acids on the depression of urinary nitrogen and creatinine excretion revealed that methionine plays a leading role in the depression of urinary nitrogen when fed as the sole nitrogenous supplement to a low protein diet.

Since these investigators found that tryptophan, phenylalanine and valine have a negligible influence on the sparing of body nitrogen, the present authors chose to work initially with tryptophan to explore, in general, its effect on urinary nitrogen excretion, especially at levels of tryptophan intake above the minimum level required for maintenance by adult rats. Since tryptophan, in contrast to methionine, causes little body-sparing action, it was thought that at high

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levels of tryptophan intake there might be an increase in the excretion of urinary nitrogen over that excreted at the minimum level of tryptophan intake, due to an excess of tryptophan in the diet.

Specifically, the present investigation involved a study of the effects of different levels of tryptophan intake upon urinary nitrogen, amino acid excretion, and nitrogen balance. We were especially interested in determining if excessive amounts of tryptophan would increase the excretion of nitrogen and of certain amino acids.

EXPERIMENTAL

Adult, male albino rats of the Sprague-Dawley strain were used in three different experiments. Each experiment consisted of several periods. During the first phase the rats received a protein depletion diet for 12 days, and urinary collections were made during the last 5 days of this period (period 1). The succeeding phases consisted of protein feeding or maintenance periods of 5 days each: 12th to 17th day on experiment, period 2; 17th to 22nd day, period 3; 22nd to 27th day, period 4. Experiments 1 and 3 were terminated at the end of period 3 (22 days) and experiment 2 at the end of the 4th period (27th day). Collections of urine and feces were made during each period.

In experiment 1, 12 rats weighing 247 ± 18 [2] gm (Frazier et al., '49) were used. Before the protein depletion phase, the rats were maintained for two to 6 weeks on an 18% casein diet. For experiment 2, 15 rats weighing 196 ± 20 [3] gm were used. These animals received a high fat diet (10 to 35% corn oil) containing 20% casein previous to the 12-day depletion period. The 15 rats used in experiment 3 weighed 204 ± 5 [4] gm. They were fed a standard stock ration for several days before receiving the protein depletion diet. At the end of the 12-day protein depletion phase the animals of experiment 1

[2] Standard deviation from the mean.
[3] See footnote 2.
[4] See footnote 2.

weighed 194 ± 24 gm, those of experiment 2 weighed 163 ± 14, and those of experiment 3, 147 ± 5.6 gm.

The composition of the basal ration used in experiment 1 is given in table 1. The basal ration for experiments 2 and 3 differed from this in that the level of acid hydrolyzed casein plus added amino acids was one-half that for experiment 1. In experiments 2 and 3 the levels of amino acids, except tryptophan, were made to conform as closely as possible to those amounts reported by Cannon [5] to be necessary for the maintenance of adult, male albino rats. In experiment 1 the ration was supplied ad libitum. In experiments 2 and 3 the food consumption was kept as close as possible to 8.0 gm per day per rat, and supplied amounts of the essential amino acids close to the levels necessary for maintenance of a 200 gm rat.

The levels of tryptophan are indicated in table 2, first column, as well as in figure 2. Each level was fed to three animals. L-tryptophan was substituted for DL-tryptophan in experiments 2 and 3, although there is good evidence that DL-tryptophan is as active as the L-form for the rat (Junqueira and Schweigert, '48).

Specially constructed metal cages [6] with removable wire bottoms and tops, permitting easy access for cleaning and care of the animals, were utilized for the metabolism experiments. Wire tunnels attached to openings in the sides of the cages allowed passageway to the ration which was placed in detachable containers constructed with wire screening (no. 4 mesh) covering the ration. This arrangement permitted the animals to reach the ration only by pushing their tongues between the spaces in the mesh, which was itself accessible only through a circular hole just large enough for the rat's head. In this manner contamination of the urine and feces due to the dropping of particles of the ration from fur and paws

[5] P. R. Cannon, Pathology Department, University of Chicago. See: Frazier, L. E., R. R. Woolridge, C. H. Steffee and E. P. Benditt 1949 Fed. Proc., *8:* 355.

[6] Modification of cages designed by Dr. J. A. Miller, McArdle Memorial Laboratory, University of Wisconsin.

TABLE 1

Basal ration for experiment 1 [1]

COMPONENTS	AMOUNT
	%
Casein (acid hydrolyzed) [2]	4.30
Amino acid mixture [3]	3.26
Vitamin mixture [4]	2.00
Salts IV [5]	4.00
Corn oil	5.00
Sucrose to make 100 gm, plus levels of tryptophan (see fig. 2).	

[1] The basal diet was supplemented weekly with two drops per rat of fortified Abbott's Haliver oil. The oil contained: Vitamin A, 600,000 U.S.P. units/gm oil; vitamin D, 600 U.S.P. units/gm oil; vitamin E, 5.0 gm/100 ml oil; vitamin K, 400 mg/100 ml oil.

[2] The acid hydrolyzed casein was prepared from Smaco casein by a method described by Henderson, Deodhar, Krehl and Elvehjem ('47). Average percentage values for the essential amino acids and arginine in two separate preparations of acid hydrolyzed casein, as determined by the microbiological method, were as follows: leucine, 8.55%; valine, 6.20%; phenylalanine, 3.80%; histidine, 1.84%; lysine, 5.90%; isoleucine, 5.05%; methionine, 2.68%; threonine, 3.25%; arginine, 2.83%; tryptophan, 0.00%.

[3] Amino acid mixture: DL-phenylalanine, 0.265%; DL-valine, 0.724%; DL-isoleucine, 1.130%; L-leucine, 0.115%; DL-threonine, 0.560%; DL-methionine, 0.400%; L-histidine monohydrochloride, 0.064% — total 3.26%.

[4] Vitamin mixture (weight per 100 gm ration): thiamine hydrochloride, 200 μg; riboflavin, 300 μg; nicotinic acid, 1.5 mg; pyridoxine, 250 mg; calcium pantothenate, 2.0 mg; biotin, 10 μg; choline chloride, 100 mg; *i*-inositol, 10 mg; pteroylglutamic acid, 20 μg; plus sucrose to make 2.0 gm.

[5] Phillips and Hart ('35).

TABLE 2

The effect of tryptophan ingestion on the excretion of tryptophan in the urine
(Experiment 3, period 3)

	TRYPTOPHAN		
Levels of intake	Excreted	Ingested	$\frac{\text{Excreted}}{\text{Ingested}} \times 100$
mg/100 gm ration	*mg/rat/day*	*mg/rat/day*	
0	0.125	0	...
50	0.194	4.2	4.62
100	0.188	8.2	2.29
150	0.190	11.9	1.60
250	0.274	20.5	1.37

was kept at a minimum. As a further precaution, the whiskers of the animals were clipped at the beginning of each experiment. In each of the three experiments the rats were allowed to become accustomed to the method of feeding (tongue-feeding) and the cage arrangement during the first 7 days of the protein depletion phase before any urine or feces collections were made.

Fresh ration was furnished each day and the amount of food consumed was weighed to the nearest 0.1 gm. Tap water was regularly supplied from bottles fastened to the outside of the metabolism cages. The weight of each animal was recorded every three days and at the beginning and end of each collection period.

The feces collection for each rat was made on an inverted cone-shaped wire screen, which was fitted into a 6-inch glass funnel serving as a catch and drain beneath each cage for the urine. Urine collections were preserved with toluene and hydrochloric acid. At the start of every collection period each flask received 2.5 ml of 2 N HCl to prevent loss of nitrogen as ammonia.

At the termination of each period the funnels were washed with distilled water and the urine filtered and diluted to the desired volume (usually 250 ml). Aliquot samples (10 ml) supplying approximately three to 15 mg of nitrogen were taken for analysis by the modified semi-micro Kjeldahl method of Chibnall et al. ('43).

In experiment 1 the feces were freed of extraneous matter, dried at 110°C. for one to two hours, pooled and analyzed for total nitrogen. Later, in experiments 2 and 3, the feces were collected daily in test tubes containing 10 to 15 ml of 95% alcohol and one drop of concentrated H_2SO_4. Just before analysis for nitrogen the alcohol was evaporated off and the feces ground and sampled, and the samples analyzed for nitrogen. This latter method was found to be more convenient and satisfactory than the former procedure.

In addition to the analysis for nitrogen in the urine of the animals of experiment 3, period 3, analysis by the microbio-

logical method (Henderson and Snell, '48) for 14 amino acids was undertaken, to determine the amount of change of the amino acids in the urines of the rats fed the differing levels of L-tryptophan under the conditions of the experiment. The methods of preparation and assay of urine samples were similar to those of Bhatt ('48).

In brief, the urines were evaporated to dryness under vacuum and the residue taken up in a few milliliters of distilled water. Four milliliters of 3 N HCl were added for acid hydrolysis of the urines and 2 ml of 5 N NaOH for alkaline hydrolysis. Minimum amounts of the acid and base were used to prevent possible salt inhibitions in the microbiological assay of the hydrolyzed samples. The acid urines were autoclaved for 5 hours at 15 lb. pressure, and the alkaline urines for 12 hours at the same pressure. Both types of hydrolysates were made up to 50 ml of solution, from which appropriate dilutions were made for estimation of their amino acid content by the microbiological assay method.

DISCUSSION AND RESULTS

In figure 1 the total nitrogen balance for experiment 2, periods 2 and 4, is plotted against the milligrams of L-tryptophan fed per 100 gm ration.

In figure 2 the milligrams of urinary nitrogen excreted per day (average for three animals) are plotted against amounts of DL- or L-tryptophan per 100 gm ration. In each experiment represented the urinary nitrogen excretion decreased to a nearly constant value at a level of supplementary tryptophan slightly above that which gave positive nitrogen balance (fig. 1).

The shape of the nitrogen excretion curves for experiment 1 is essentially the same as for experiments 2 and 3 except for the higher excretion of nitrogen in experiment 1, which was probably due to the higher level of dietary protein in this experiment. The plateau in the curves consistently began between the 50 and 100 mg levels of tryptophan. The curves plotted were essentially flat in all cases between the 100 and

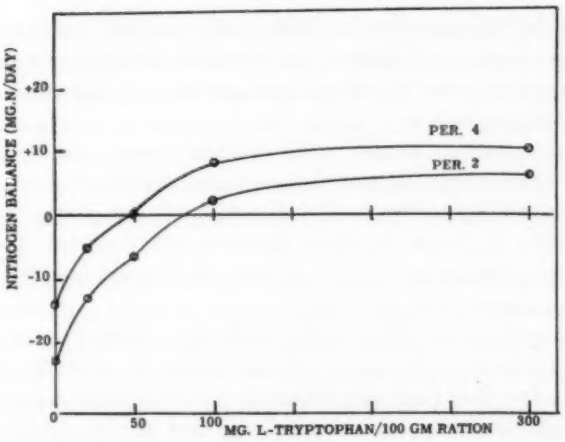

Fig. 1 Total nitrogen balance of experiment 2, periods 2 and 4, plotted against levels of L-tryptophan ingested per 100 gm ration. Each point represents averaged data from three animals.

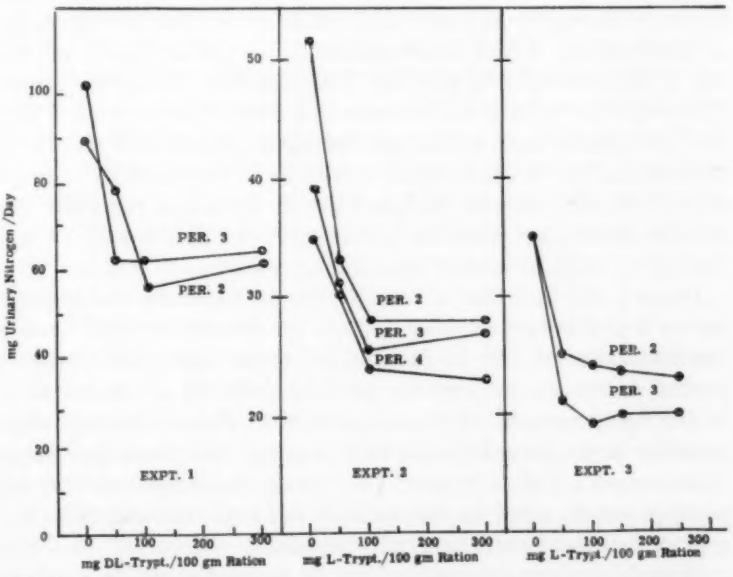

Fig. 2 The average urinary nitrogen excretion per day of experiments 1, 2 and 3 plotted against levels of tryptophan ingested per 100 gm ration. Each point represents averaged data from three animals.

300 mg tryptophan levels. This indicates that as the amount of tryptophan rose above the minimum level to an excess in the diet there was no detectable increase in the nitrogen excretion in the urine.

In experiments 2 and 3 (fig. 2) the curves for milligrams of nitrogen excreted per day against milligrams of tryptophan per 100 gm of ration lie one below the other in the order of periods 2, 3 and 4. This indicates that as the length of time the animals were on a given diet increased, less nitrogen was excreted in the urine.

In experiment 2 (fig. 1) the difference between the curves representing the nitrogen balance data for periods 2 and 4 appears to reveal an alteration in the minimum requirement for tryptophan as the animals adjusted to the diets. On the other hand, the animals may have required less of the amino acid as their weights decreased or as their metabolic needs were altered in some manner. In experiment 2, period 4, the positive nitrogen balance was initiated at the 50 mg level of tryptophan, which corresponds to a consumption of 4.0 mg of this amino acid per day. This amount is slightly below the 4.8 level (calculated) recommended by Frazier et al. ('49) for the maintenance of 200 gm rats. An inclusion of 60 mg of tryptophan per 100 gm ration would have supplied 4.8 mg per day. However, the rats weighed nearly 25% less than 200 gm at this particular point in the experiment and would be expected to require less of this amino acid.

Despite the fact that all conditions and animals for experiments 2 and 3 were similar, except for the rations fed before the initiation of the 12-day depletion periods, the nitrogen excretion for corresponding periods differed by about 15% in the early periods of the experiments. This difference may possibly be explained by the fact that the rats for experiment 3 consumed a high nitrogen diet (stock diet) prior to the depletion period, whereas the animals for experiment 2 received a high fat diet containing 20% casein.

Microbiological determinations of the amino acids proline, threonine, leucine, histidine, and methionine in acid hydro-

lyzed urine, and tryptophan in alkaline hydrolyzed urine (experiment 3, period 3), revealed a somewhat consistent pattern in that all these amino acids were found to be excreted in nearly constant amounts at all levels of tryptophan intake.

Our data also indicate that there was a variable increase in the excretion of the dispensable amino acids aspartic acid, glutamic acid, and glycine as the level of dietary tryptophan increased.

Table 2 summarizes the data for experiment 3, period 3, on the relationship between tryptophan ingestion and excretion per day when the basal diet contained increasing levels of L-tryptophan. The results are in good agreement with the work of Sauberlich et al. ('46, '48) and Schweigert ('47) on tryptophan excretion by the rat.

SUMMARY

1. Protein-depleted, adult rats were fed low protein diets containing tryptophan-free acid hydrolyzed casein supplemented with the other essential amino acids to render the diet adequate for maintenance. The feeding of supplementary levels of tryptophan, ranging from zero to 300 mg per 100 gm of ration, caused a marked decrease in urinary nitrogen excretion up to a point between the 50 mg and 100 mg levels. Amounts of tryptophan ranging from 100 mg to 300 mg (8 to 24 mg per rat per day) did not appreciably alter the level of excretion of urinary nitrogen.

2. The requirement for tryptophan of adult rats fed a low protein diet supplying a minimum of the essential amino acids for maintenance and some of the non-essential amino acids, varied from 4.0 mg to 6.4 mg per 200 gm rat per day, depending upon the length of time that elapsed after feeding the protein depletion diet.

3. The feeding of minimum levels and twice the minimum levels of the amino acids required for maintenance, in the presence of graded levels of tryptophan, resulted in a similar pattern of nitrogen excretion.

4. When the daily food consumption was approximately 8.0 gm per day, positive nitrogen balance was possible in adult rats which received a diet containing only 2.9% protein.

5. Excessive amounts of tryptophan in the diet caused little change in the urinary excretion of tryptophan, threonine, leucine, histidine, methionine, arginine and proline.

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A COMPARISON OF PARENTERALLY AND ORALLY SUPPLIED PROTEIN HYDROLYSATE FOR UTILIZATION OF THE NITROGEN IN LONG CONTINUED FEEDING EXPERIMENTS

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INTRODUCTION

The oral or parenteral administration of protein hydrolysates, as reported by Elman ('44), Allison and co-workers ('46), and Chow and associates ('48b) has been observed experimentally and clinically to promote the regeneration of plasma protein and to maintain a positive nitrogen balance.

Protein nutrition studies, wherein experimentally depleted animals were fed by either parenteral (Elman, '44) or oral routes (Chow, Alper and DeBiase, '49), have shown that approximately 50% of the administered nitrogen is retained by either route. Parenteral feeding was carried out during a short experimental period.

Clark, Brunschwig and Corbin ('42) reported that regeneration of plasma proteins occurred in protein depleted dogs receiving via the intravenous route an enzymatic hydrolysate of casein as the sole source of nitrogen. The injection periods in 8 experiments varied from 7 to 33 days. Most of the experiments were of about 15 days' duration. They yielded no in-

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formation on the completeness of regeneration of protein in the protein depleted dogs by prolonged parenteral feeding of the hydrolysate. Did nitrogen retention and plasma protein regeneration reach a maximum level? Would further nitrogen retention and plasma protein regeneration occur if the mode of nitrogen administration were altered after nitrogen saturation by the parenteral route?

If dogs depleted of their protein reserves were fed protein hydrolysate parenterally for a prolonged period, followed by a similar period of oral hydrolysate feeding, a comparison of the values for nitrogen retention and plasma protein regeneration during the two periods would be more indicative of differences in the extent of utilization of the administered nitrogen as a function of the feeding pathway than experiments using only one such pathway. Such an experiment would provide information concerning the concentration of hydrolysate N in the animal's circulatory system which can be efficiently metabolized. Parenteral feeding of protein hydrolysate rapidly presents the animals with a high level of hydrolysate nitrogen, whereas oral feeding of protein hydrolysate presents the animal with a level of hydrolysate nitrogen controlled by the physiological mechanism of intestinal absorption. This experiment would also shed light on the qualitative differences between tryptic digests of casein administered by the intravenous route and tryptic digests of casein further digested by the systemic proteolytic enzymes of the experimental animals. In such an experiment, one pattern of amino acids and peptides is presented to the body for utilization in tissue synthesis during severe nitrogen depletion; a second pattern is presented to the body after nitrogen saturation by parenteral hydrolysate feeding. Can this qualitative difference be detected?

To this end, the experiment outlined below was undertaken. Mongrel dogs were experimentally depleted of plasma proteins. They were fed for a prolonged period with casein hydrolysate, parenterally; then they were fed for an additional period with casein hydrolysate, orally, to ascertain nitrogen

retention and plasma protein regeneration during both phases of the experiment. The results of our investigation are presented in this paper.

EXPERIMENTAL

Depletion of protein reserves

Adult mongrel dogs of average weight (7 to 12 kg) were made hypoproteinemic (a) by feeding a protein-free diet (Weech et al., '33), or (b) by feeding a protein-free diet coincident with plasmapheresis (Seeley, '45).

Allison and Anderson ('45) described the protein-free diet used in these experiments. When the casein hydrolysate diet replaced the protein-free diet, casein hydrolysates replaced glucose isocalorically.

The daily diet of each dog furnished about 80 cal. per kilogram of body weight per day. Depletion of protein stores on a protein-free diet at this caloric intake requires 6 to 8 weeks (Chow, Alper and DeBiase, '48b).

Dogs depleted by protein-free feeding concomitant with plasmapheresis were fed a protein-free diet during the first week. During the second week, plasmapheresis was performed every other day for three days. Approximately one-quarter of the total blood volume, estimated on the basis of body weight, was removed, and washed red cells were returned in an equivalent volume of physiological saline.

Several of the dogs extensively depleted by protein-free feeding showed some of the external symptoms of protein deficiency; pressure sores and edema of the hind and fore extremities. Edema disappeared shortly after hydrolysate therapy commenced; pressure sores gradually healed in several weeks. All of the depleted dogs showed a marked lassitude.

Six dogs (group 1) were gradually depleted by protein-free feeding at an essentially constant caloric intake over a period of 6 to 8 weeks. Four dogs (group 2) were depleted by protein-free feeding with concurrent plasmapheresis. The average urinary nitrogen of the depleted dogs of group 1 was 0.066 ± 0.012

gm/kg/day, whereas that of the depleted dogs of group 2 was 0.117 ± 0.019 gm/kg/day. The average normal value was about 0.120–0.130 gm/kg/day. The average plasma protein concentration for the depleted dogs of group 1 was 4.43 ± 0.29 gm %, and the average value for the depleted dogs of group 2 was 4.75 ±0.52 gm %. The average normal value was 5.9–6.4 gm %. The plasma protein concentration was in the same range in both groups of depleted animals, but the urinary nitrogen excretion of the dogs of group 1 was not quite one-half that of the dogs of group 2. These results demonstrated that plasmapheresis causes immediate depletion of plasma proteins but does not generally disturb the protein reserves.

Repletion of protein reserves

Repletion of protein reserves was effected by parenteral feeding of 0.35 gm of casein hydrolysate nitrogen per kilogram of body weight for about three weeks. Following completion of this regime, the dogs were fed casein hydrolysate orally at the same level of nitrogen intake for a like period. Four of the dogs were fed via the intravenous route for a second period of 9 days. Water was fed ad libitum.

While on the intravenous regime, the dogs were fed a protein-free diet orally. Several of the animals did not eat this part of the diet completely while on the intravenous regime. Consequently their caloric intake was below the norm of 80 cal. per kilogram of body weight.

Parenteral infusion by gravity was continuous for one to two hours in the veins of the hind extremities. Dogs were strapped to a table, and alternate legs were used each day. At this rate (approximately 40 mg N/kg/min.), there were no symptoms of discomfort. On several occasions the dogs vomited on the first and second days of the three-week series of infusions. If care was taken with the injections, none of our dogs suffered from serious thrombosis of the veins.

The repletion regime is summarized in table 1. Both groups of dogs were maintained under the same controlled conditions.

Preparation of casein hydrolysate [3]

Sixteen hundred grams of freshly precipitated casein were suspended in 7 l of pyrogen-free distilled water. Approximately 100 ml of 4 N NaOH were added to bring the pH of the solution to 7.6. Four grams of purified trypsin were added to the solution. Ten milliliters of toluene were added as a preservative. After 24 hours of digestion at 55°C., 3.0 gm of carboxypeptidase were added and the mixture was again incubated at 55°C. for about two days. The suspension was then autoclaved at 15 lb. pressure for 15 min. and filtered with an Ertel filter. The filtrate was found to be pyrogen-free and to contain 13.5 mg N per milliliter, of which 40% was alpha amino nitrogen as determined by the ninhydrin method (Van Slyke, MacFadyen and Hamilton, '43).

TABLE 1

Repletion regime of depleted dogs

GROUP NO.	NO. OF DOGS	PARENTERAL HYDROLYSATE			ORAL HYDROLYSATE		
		Days	Caloric intake	N intake	Days	Caloric intake	N intake
				gm/kg/day			*gm/kg/day*
1	6	25	73 ± 23	0.349 ± 0.016	24 [1]	81 ± 6 [1]	0.363 ± 0.037 [1]
2	4	24	87 ± 6	0.342 ± 0.007	21	78 ± 2	0.350 ± 0.011

[1] One of the dogs died; average of 5 dogs.

Nitrogen retention and plasma protein regeneration

Urine collections were made at three- and four-day intervals throughout the duration of the experiment. One representative fecal collection of 7 days' duration was made during each feeding period. The feces during the protein-free feeding period were soft and fatty, while during the parenteral or oral hydrolysate feeding periods they were of normal consistency.

[3] The casein hydrolysate for oral use was prepared according to the directions of Chow, Allison and White ('48a).

The methods used for the determination of plasma volume and plasma protein concentration and for the estimation of plasma components have been described by Chow, Seeley, Allison and Cole ('48c).

RESULTS

Alteration in body weight

The dogs on a protein-free diet generally lost body weight. Part of this loss was body protein stores. Parenteral infusion of casein hydrolysate to depleted dogs maintained the dogs at relatively constant weights. For the entire period of parenteral hydrolysate feeding, the dogs of group 1 exhibited an average loss of 0.1 kg; the animals of group 2 exhibited an average gain during this period of 0.2 kg. During the period of oral hydrolysate feeding the dogs of group 1 showed an average gain of 0.9 kg; those of group 2, a gain of 0.5 kg.

Nitrogen balance

Nitrogen retention can be expressed as the value obtained by subtracting total nitrogen excretion while on the test diet from the sum of the N intake and urinary and fecal nitrogen excretion on a protein-free diet.

The summary of the nitrogen balance experiments presented in table 2 demonstrated that severely depleted dogs were in more positive nitrogen balance than those subjected to plasmapheresis. However, the over-all nitrogen retention was about the same for the both groups of animals.

Dogs were maintained in positive N balance throughout the experiment. However, during the oral feeding period, following partial repletion by parenteral infusion of hydrolysate nitrogen, the average nitrogen retention coefficient was 1.7. The nitrogen retention coefficient may be defined as the ratio of the per cent nitrogen retained by the oral route of administration to the per cent nitrogen retained by the parenteral route. The individual values for the dogs in group 1 were 1.6,

TABLE 2

Summary of nitrogen utilization studies

GROUP NO.	NO. OF DOGS	NITROGEN BALANCE		NITROGEN RETENTION		RETENTION		GM PROTEIN/KG/CAL.	
		Intravenous	Oral	Intravenous	Oral	Intravenous	Oral	Intravenous	Oral
		gm	*gm*	*gm*	*gm*	%	%		
1	6	+ 0.071 ± 0.023	+ 0.165 ± 0.027	+ 0.131 ± 0.015	0.230 ± 0.038	36.6	63.3	1.8 ± 0.58 [1]	3.5 ± 0.45 [2]
2	4	+ 0.023 ± 0.018	+ 0.145 ± 0.015	+ 0.101 ± 0.021	0.222 ± 0.018	43.7	84	0.38 ± 0.33 [3]	2.5 ± 0.5 [4]

[1] Individual values — 1.8, 0.99, 2.1, 2.3.

[2] Individual values — 0.5, 0.02, 0.20, 0.80.

[3] Individual values — 3.1, 3.5, 4.1, 3.2.

[4] Individual values — 1.8, 2.8, 2.9, 2.4.

1.7, 1.7, 1.7, and 1.9. Those for the dogs in group 2 were 1.8, 2.5, 1.6, and 1.7. Values were not constant in the second group because of the poor retention of nitrogen during the parenteral hydrolysate feeding of dogs depleted rapidly of protein reserves by plasmapheresis and protein-free feeding.

As a measure of the degree of depletion, it was interesting to note the grams of plasma protein repleted per kilogram per calorie. Values were very constant when the hydrolysate was given orally but rather erratic when it was given parenterally (see table 2).

Plasma protein regeneration

The level and distribution of plasma proteins might be considered an index of the state of protein nutrition of the experimental animal. The results of these experiments showed that, although the oral administration of casein hydrolysate showed better utilization by the animal previously repleted parenterally, a greater percentage of nitrogen was diverted for plasma protein regeneration during parenteral hydrolysate feeding when depletion was most severe. In group 1, 6.7% of the nitrogen administered by the parenteral route and 2.2% of the oral nitrogen was diverted to plasma protein regeneration. In group 2, more than 2% of the parenteral nitrogen was directed to the locus of plasma protein synthesis and 1.7% of the oral nitrogen was utilized for this purpose. However, it should be pointed out that the actual increase in plasma protein was greater following oral administration. Table 3 summarizes the results of these experiments.

Second period of intravenous hydrolysate feeding

The observation of the increased retention of nitrogen during oral feeding of casein hydrolysate after a 24- to 25-day repletion period by casein hydrolysate supplied parenterally stimulated interest in the nitrogen retention which would result from a second period of parenteral administration of the hydrolysate after completion of the oral feeding period. The

dogs were infused for 9 days. Only 25% of the administered nitrogen was retained during this period. There was no alteration in the plasma protein picture.

DISCUSSION

In contrast to the observations made on small laboratory animals such as rats, mice and guinea pigs, the repletion of depleted dogs cannot be expressed conveniently in terms of

TABLE 3

Plasma protein regeneration

NUMBER OF DOGS	GROUP 1 5	GROUP 2 4
Plasma protein concentration (gm %)		
(a) Depleted dogs	4.43 ± 0.29	4.75 ± 0.52
(b) Post intravenous feeding	5.00 ± 0.38	4.93 ± 0.24
(c) Post oral feeding	5.86 ± 0.22	5.75 ± 0.35
Plasma volume (ml)		
(a) Depleted dogs	548 ± 101	467 ± 113
(b) Post intravenous feeding	611 ± 116	488 ± 120
(c) Post oral feeding	635 ± 155	480 ± 112
Total circulating protein (gm)		
(a) Depleted dogs	24.07 ± 2.92	22.38 ± 5.66
(b) Post intravenous feeding	30.69 ± 6.92	24.17 ± 6.48
(c) Post oral feeding	36.99 ± 7.8	27.62 ± 6.84

gain in body weight, since body weight increment is small compared to total body weight. There is a definite trend toward body weight increase after the oral feeding of casein hydrolysate. Repletion by casein hydrolysate supplied parenterally showed no significant trend in the alteration of body weight in either direction. This might be explained by the fact that of the 35% nitrogen retained, about 6% is diverted to plasma protein regeneration. The remainder goes to tissue protein, but the total quantity is so little that the over-all weight of the dog is not altered.

In hypoproteinemia effected by restricted plasmapheresis, the "reserve protein stores" of the experimental animal are not permanently depleted, although plasma protein concentration and plasma volume are reduced. On the other hand, feeding an experimental animal a protein-free diet over a long period of time will drain the "reserve protein stores" and finally deplete the plasma proteins. Animals depleted by the latter method, where there is extensive loss of tissue proteins, might be expected to show different utilization of the parenteral nitrogen supplement than the animal not so "completely" depleted.

The present experiments demonstrate that severely depleted animals may be maintained in positive nitrogen balance through the entire period of parenteral infusion of casein hydrolysate.

After both groups of animals were partially repleted parenterally by protein hydrolysate they showed no significant difference in their retention of nitrogen on an oral hydrolysate diet.

It is interesting indeed to observe that, although the dogs had been partially repleted by parenteral feeding, about twice as much nitrogen was retained during the oral feeding period as during the parenteral feeding, in spite of the greater increase in plasma protein after oral feeding. Perhaps the pattern of amino acids and peptides presented to the animal by normal peptic and tryptic digestion is better utilized than amino acids and peptides resulting solely from tryptic digestion *in vitro*. Chow ('48) recently reported that the type of hydrolysis of casein does not influence the nutritive index of casein hydrolysate. Yet one must remember that for all practical purposes casein hydrolysates administered orally are always subjected *in vivo* to peptic and tryptic digestion. Had the casein hydrolysates prepared by digestion of casein with fungal and bacterial proteases been administered parenterally, one might have observed entirely different results. The obobservation of extremely high retention of orally administered nitrogen after partial repletion by parenteral nitrogen ad-

ministration may also be explained by the concentration of peptides and amino nitrogen in the material presented to the animal and its particular needs at that time.

Plasma proteins are repleted by either method of administering the hydrolysate. A greater percentage of the nitrogen retained during parenteral feeding is diverted to plasma protein repletion, regardless of the fact that by the oral route of administration almost twice as much nitrogen is retained. This may be explained by the high concentration of nitrogen presented to the animal in a form which may be used directly for synthesis of plasma protein, or by the nature of the polypeptide presented to the sites of plasma protein synthesis. Hence, the parenteral administration of hydrolysate serves to fill the immediate requirements of the experimental animals, whereas oral feeding serves their long term needs.

The lack of change in A/G ratio during repletion would seem to indicate that the rate of repletion of albumin and globulin is constant under these conditions.

Although the severely depleted dogs were maintained in positive nitrogen balance by parenteral infusion of casein hydrolysate for long periods of time, repletion of the protein stores of the animal apparently was not complete. Alteration of the route of nitrogen administration resulted in significantly increased nitrogen retention, with more nitrogen being diverted to the repletion of depleted protein stores.

SUMMARY

Dogs were depleted of protein reserves by the feeding of protein-free diets with and without the accompaniment of plasmapheresis. Repletion was carried out by parenteral infusion of casein hydrolysate for an extended period, followed by a like period of oral feeding of casein hydrolysate. During repletion the dogs showed no striking increments in body weight. Even though partial repletion had been effected by intravenous infusion of casein hydrolysate, the dogs retained more nitrogen via the oral route than via the parenteral route.

The nitrogen retention coefficient of the dogs was 1.7. The gain in protein per kilogram of body weight per calorie of intake via the oral route is twofold the value obtained via the parenteral route in animals subjected to extensive depletion. More of the nitrogen retained via parenteral feeding was utilized for plasma protein regeneration than of that retained by oral feeding, although more total nitrogen was retained by the oral route of administration.

There appeared to be a difference in the utilization of administered nitrogen dependent upon the extent of depletion of the "protein reserves" of the experimental animal and the mode of administration of the hydrolysate. A possible explanation for this difference has been discussed.

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INTERRELATIONSHIP OF FOLACIN, VITAMIN B_{12} AND CHOLINE

EFFECT ON HEMORRHAGIC KIDNEY SYNDROME IN THE RAT AND ON GROWTH OF THE CHICK [1]

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SEVEN FIGURES

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It has been established in this laboratory that the choline requirement of the rat and chick may be significantly decreased by supplementation of the diet with vitamin B_{12} (Schaefer, Salmon and Strength, '49a, '49b). The studies have been extended to determine whether folacin has a choline-sparing effect and whether vitamin B_{12} and folacin have a sparing effect on each other. It will be reported in this paper: (1) that the incidence and severity of renal damage in weanling rats receiving a diet low in choline and methionine were significantly decreased by supplementing the diet with folacin, and were further decreased when the diet was supplemented with both folacin and vitamin B_{12}; (2) that the chick has a definite requirement for choline, folacin, and vitamin B_{12} and that these nutrients are interrelated in furthering growth, hemoglobin formation, feathering, and the prevention of perosis.

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EXPERIMENTS WITH RATS

Weanling rats of the Alabama Experiment Station (AES) strain weighing 40 to 50 gm were placed in individual cages and uniformly grouped with respect to number, weight, sex and litter. Feed and water were supplied ad libitum. The

TABLE 1

Basal diets[1]

COMPONENTS	RAT DIET	CHICK DIET
	gm	*gm*
Extracted peanut meal[2]	300	300
Sucrose	395	369
Extracted casein[3]	60	60
Salts	44[3]	70[4]
L-cystine	1	1
Cod liver oil	10	10
Lard	190	190
	mg	*mg*
Thiamine	2	3
Pyridoxine	2	4
Riboflavin	4	6
Ca pantothenate	10	20
Niacin	20	50
Inositol	200	1000
α-tocopherol	25	25
α-tocopherol acetate	25	25
2-methyl-1,4-naphthoquinone	5	5
Biotin	..	0.25

[1] Total methionine content of diets was 0.3%; total choline content of diets was 0.007%.

[2] Engel, R. W., J. Nutrition, 1948, *36:* 739.

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composition of the basal diet is given in table 1. All rats were necropsied after death or at the end of a 14-day experimental period, and the kidneys were carefully examined for gross pathological lesions. The results are summarized in table 2. In rats receiving the basal diet supplemented with 0.04% of choline chloride or 0.128% of DL-methionine there

was 100% incidence of renal hemorrhage. The addition of folacin at levels of 2, 10, and 100 mg per kilogram of basal diet supplemented with 0.04% choline chloride reduced the incidence of kidney damage to 75, 42 and 50%, respectively. The incidence of kidney hemorrhage was reduced to zero by the addition of 10 mg folacin and 30 μg vitamin B_{12} per kilogram to the basal diet supplemented with 0.04% choline chloride or 0.128% DL-methionine.

TABLE 2

Choline-sparing action of folacin and vitamin B_{12} as measured by protection against renal damage in rats (two-week experimental period)

DIETARY SUPPLEMENT			NO. RATS	MORTALITY	AVG. WT. GAIN OF SURVIVORS	INCIDENCE RENAL DAMAGE
Choline chloride	Folacin	Vitamin B_{12}				
%	*mg/kg*	*μg/kg*		%	*gm*	%
0.04	0	0	37	21.6	20	100
0.04	2	0	8	0.0	43	75
0.04	10	0	12	0.0	53	42
0.04	100	0	4	0.0	50	50
0.04	0	Conc. no. 4 ≎ 150 [1]	4	0.0	51	50
0.04	10	Conc. no. 1 [2] ≎ 30	12	0.0	71	0
0.04	10	Conc. no. 4 ≎ 30	4	0.0	67	0
0.02	10	Conc. no. 4 ≎ 30	12	8.3	45	50
0.00	10	Conc. no. 4 ≎ 30	4	25.0	14	100
DL-Methionine						
0.128	0	0	4	50.0	2	100
0.128	10	Conc. no. 4 ≎ 30	4	0.0	69	0

[1] One gram vit. B_{12} conc. no. 4 = 56.4 μg vit. B_{12}.
[2] One gram vit. B_{12} conc. no. 1 = 4.3 μg vit. B_{12}.

Increasing the vitamin B_{12} to 150 μg without folacin did not reduce the incidence of kidney hemorrhage below the 50% that was previously reported at the 30 μg level (Schaefer et al., '49a). The choline-sparing action of vitamin B_{12} and folacin seems to be an additive effect, since very high levels of either folacin or vitamin B_{12} are unable to replace each other completely.

In the rats receiving either folacin or vitamin B_{12} renal damage was mild in comparison with that in the control rats

not receiving these nutrients. No deaths occurred among the 44 rats receiving 0.04% choline chloride plus folacin or vitamin B_{12}, or both. The average weight gain for the two-week period ranged from 43 to 53 gm for those receiving either folacin or vitamin B_{12} and from 67 to 71 gm for those receiving both nutrients. On the other hand, 8 deaths occurred among 37 rats fed the basal diet supplemented with 0.04% choline chloride without further supplements, and the average weight gain of the survivors was only 20 gm.

Previous studies showed 100% incidence of renal damage in rats fed 0.192 and 0.150% of supplementary DL-methionine. However, when the diet was further supplemented with vitamin B_{12}, the incidence of kidney damage decreased to 12% at the 0.192% level of methionine and to 25% at the 0.15% level (Schaefer et al., '49a). The addition of both folacin and vitamin B_{12} to the diet supplemented with only 0.128% DL-methionine afforded complete protection against renal hemorrhage and supported maximum growth.

EXPERIMENTS WITH CHICKS

Day-old Leghorn chicks of the Auburn strain were divided into three weight classes ranging from 34 to 42 gm. The selected chicks were then distributed, 8 to 15 per group, so that the average weight in each group was about 38 gm. The chicks were wing-banded and housed in standard commercial electrically-heated batteries equipped with raised wire floors. Feed and water were supplied ad libitum daily and the chicks were weighed weekly. The composition of the basal diet is given in table 1.

Effect on growth

The results of supplementing the basal diet with 0.60% or 0.10% choline chloride and varying levels of folacin or vitamin B_{12} or both are given in table 3. When the basal diet was supplemented with 0.60% choline chloride, the addition of either 30 μg vitamin B_{12} or 2 mg folacin per kilogram of

diet caused an average increase in weight gain at 4 weeks of 70 and 67 gm, respectively. Supplementation of the diet with both folacin and vitamin B_{12} concentrate resulted in a weight increment of 126 gm.

Increasing the folacin to 100 mg per kilogram of diet, without vitamin B_{12}, did not support maximum growth. Although the weight gain at 4 weeks was 16 gm greater than in the

TABLE 3

Effect of choline, folacin, and vitamin B_{12} on growth, hemoglobin, mortality, and perosis in chicks

EXP'T. NO.	DIETARY SUPPLEMENT			NO. OF CHICKS	4 WEEKS		6 WEEKS	
	Choline chloride	Folacin	Vitamin B_{12}		Avg. wt. gain/ chick	Hb	Mortality	Perosis
	%	*mg/kg*	*μg/kg*		*gm*	*gm %*	%	%
3,7,8,9	0.6	0	0	45	87	7.5	54	..
10	0.10	0	0	11	67	8.4	0	80
3,7,8,9	0.60	2.0	0	47	154	9.3	0	2
10	0.60	100.0	0	11	170	..	0	0
8,9	0.10	2.0	0	22	78	.	8	66
10	0.10	100.0	0	11	123	9.7	0	0
5,7,8,9	0.60	0.0	Conc. 1 and 4 ⇔ 30	50	157	9.3	0	13
10	0.60	0.0	Conc. 4 ⇔ 150	11	152	..	0	19
10	0.10	0.0	Conc. 4 ⇔ 30	11	121	..	9	22
10	0.10	0.0	Conc. 4 ⇔ 150	11	127	..	9	27
5,7,8,9,10	0.60	2.0	Conc. 1 and 4 ⇔ 30	92	213	9.8	0	0
8,9	0.10	2.0	Conc. 1 and 4 ⇔ 30	23	224	9.9	0	0
10	0.10	0.2	Conc. 4 ⇔ 150	11	205	..	0	0
10	0.10	2.0	Conc. 4 ⇔ 150	11	202	9.2	0	0
10	0.10	100.0	Conc. 4 ⇔ 5	11	172	..	0	0
6[1]	0.00	2.0	Conc. 1 ⇔ 30	16	65	..	0	100

[1] Diet supplemented with 1.31% DL-methionine.

chicks receiving 2 mg folacin, the gains at 6 weeks were equal. Similarly, increasing the vitamin B_{12} supplement to 150 μg per kilogram of diet, without folacin, did not further stimulate growth over that obtained with 30 μg vitamin B_{12}. This indicates that the gains in body weight due to folacin and vitamin B_{12} are independent of each other, and when both are present in the diet the resulting gain is an additive effect.

That vitamin B_{12} was the active principle in the concentrates is indicated by the growth curves shown in figure 1. Crystalline vitamin B_{12} fed at a level of 30 μg per kilogram of diet supplemented with 0.60% choline chloride caused an average increase in weight gain of 66 gm in 4 weeks, as compared to 68 gm produced by vitamin B_{12} concentrate no. 4 fed at a level of 0.054% so as to supply 30 μg of vitamin B_{12} per kilogram of diet.

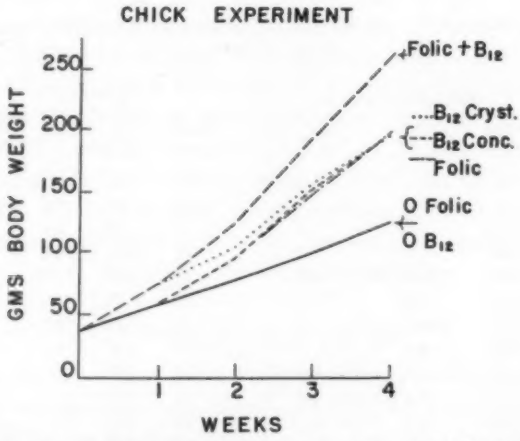

Fig. 1 Growth curves of groups of chicks (11 per group) fed the basal diet plus 0.6% choline chloride supplemented as indicated. Folacin was added at a level of 2 mg per kilogram diet; vitamin B_{12} concentrate no. 4 was fed at 0.054% = 30 μg per kilogram diet and crystalline vitamin B_{12} was supplied at 30 μg per kilogram diet.

When the basal diet was supplemented with 0.10% choline chloride, the addition of vitamin B_{12} at levels of 30 μg and 150 μg per kilogram caused an increased gain in body weight at 4 weeks of 54 and 60 gm, respectively. Folacin at levels of 2 and 100 mg per kilogram of diet resulted in a growth increment of 11 and 56 gm, respectively. The additional supplement of 5 μg vitamin B_{12} per kilogram to the 100 mg folacin, 0.10% choline chloride diet resulted in a growth increment of 105 gm. This indicates that a minute amount of vitamin B_{12}

has a pronounced effect on growth. Thus, the extra gain in body weight due to 100 mg folacin over 2 mg folacin added to the basal diet supplemented with 0.10% choline chloride may have been due either to the synthesis of vitamin B_{12} or the sparing action of folacin on the choline requirement. The dietary requirement of folacin was 0.20 mg per kilogram or less when the basal diet was supplemented with 0.10% choline chloride and 150 µg vitamin B_{12} per kilogram.

Effect on hemoglobin

When folacin and vitamin B_{12} were omitted from the diet supplemented with 0.60% choline chloride, average hemoglobin values for each experiment were 6.5, 7.4, 7.6 and 8.5 gm % at 4 weeks. The inclusion of vitamin B_{12} raised the average hemoglobin values per experiment to 9.1, 9.3, 9.5 and 9.5 gm %. In those groups receiving folacin the average hemoglobin values per experiment were 8.8, 9.3, 9.4 and 9.8 gm %. Addition of both folacin and vitamin B_{12} produced average hemoglobin levels of 9.4, 9.5, 9.9, 9.9 and 10.1 gm %. The basal diet supplemented with 0.10% choline chloride, folacin and vitamin B_{12} similarly supported normal hemoglobin levels of 9.2, 9.6 and 10.3 gm %.

Effect on perosis

The observation of an occasional case of perosis in over 300 chicks receiving the basal diet supplemented with 0.60% choline chloride and 2 mg folacin per kilogram indicated that some other nutrient was essential for complete protection against perosis. The incidence reported in table 3 represents only those cases in which grossly evident slipped tendon occurred. Chicks receiving 0.60% choline chloride without folacin or vitamin B_{12} had the highest mortality. Typical enlarged joints were not observed, but nearly 100% of the birds had a spastic paralysis. The highest incidence of perosis was noted in the birds receiving 0.10% choline chloride without folacin or vitamin B_{12}. With 0.60% choline chloride plus

vitamin B_{12} perosis was noted in 13 to 19% of the birds; an example is shown in figure 2. This high level of choline plus 2 mg folacin per kilogram of diet resulted in only an occasional case of perosis. Folacin added at a level of 100 mg per kilogram of diet in addition to 0.10% choline chloride afforded complete protection in one experiment; whereas, on a low level of folacin (2 mg per kilogram) and 0.10% of choline chloride, 66% of the chicks were perotic. In all groups receiving the basal diet supplemented with either 0.10 or 0.60% choline chloride plus both folacin and vitamin B_{12} no perosis occurred. In one experiment it was found that the diet unsupplemented with choline resulted in perosis even in the presence of adequate folacin, vitamin B_{12} and DL-methionine. Sixteen chicks were fed the basal diet supplemented with 1.31% DL-methionine, 2 mg folacin and 30 μg vitamin B_{12} per kilogram; all developed perosis. These results show that not only manganese but also folacin, vitamin B_{12} and choline are essential nutrients in protection against perosis.

A preliminary experiment on the regression of perosis in chicks was tried. Six chicks showing a moderate degree of perosis at 6 weeks, as evidenced by leg weakness and enlarged joints, were fed the basal diet supplemented with 0.10% choline chloride, 30 μg vitamin B_{12} and 10 mg folacin per kilogram. After 6 weeks on this regimen all gross signs of perosis had disappeared.

Effect on feathering

The effect of folacin and vitamin B_{12} on feathering is illustrated in figures 2, 4, 5, 6 and 7. In the absence of both folacin and vitamin B_{12} feathering was extremely poor (figs. 2 and 6) and those chicks surviving for 8 weeks or longer appeared virtually naked (fig. 5). Supplementing the diet with vitamin B_{12} greatly improved feathering; however, many shafts were incompletely feathered. The addition of folacin to the diet supplemented with 0.60% or 0.10% choline chloride produced fair to good feathering in chicks up to 8 weeks of

age (fig. 4). However, when these birds were continued on experiment for 20 weeks or longer, feathering was not normal (fig. 5). When both folacin and vitamin B_{12} were incorporated in the diet, feathering was normal (figs. 4, 6 and 7). Some of these chicks have been maintained on this diet for over 45 weeks and are in an excellent state of health and appearance. Feathering was as good in the birds receiving 0.10% choline, 0.20 mg folacin and 150 μg vitamin B_{12} per kilogram of diet as in those receiving 0.60% choline plus 2 mg folacin and 30 μg vitamin B_{12} (fig. 7).

Effect on liver and gallbladder weights

In chicks fed the basal diet plus 0.60% choline chloride in the absence of folacin and vitamin B_{12} the liver weight was 4.5% of the body weight and the gallbladder weights averaged 0.70 gm (fig. 3). Supplementing the diet with either folacin or vitamin B_{12} reduced the liver weight to 3.8% and 3.6% of the body weight, respectively. The gallbladders of the chicks appeared slightly enlarged, weighing 0.36 to 0.42 gm. In the controls receiving both folacin and vitamin B_{12} with 0.60% or 0.10% choline chloride, the liver weights averaged 2.8% to 2.2% of the body weight and the gallbladder weights averaged 0.25 to 0.22 gm.

DISCUSSION

In previous experiments (Schaefer and co-workers, '49a) it was shown that vitamin B_{12} decreases the dietary choline or methionine required for protection against the hemorrhagic kidney syndrome in rats. When vitamin B_{12} in concentrate or in crystalline form was added to the basal diet supplemented with 0.04% choline chloride, the incidence of renal damage was reduced from 100% to 50%. The results of the experiments herein reported show that folacin also affords some protection against renal damage. When both folacin and vitamin B_{12} were added to the basal diet supplemented with either 0.04% choline chloride or 0.128% methionine there

was complete protection against kidney damage. Since high levels of either folacin or vitamin B_{12} could not replace each other at this suboptimum level of choline, the sparing action of these nutrients on the dietary choline requirement seems to be an additive phenomenon. Complete protection against renal damage in the absence of vitamin B_{12} and folacin requires the addition of either 0.10% choline chloride or 0.48% DL-methionine to the basal diet (Engel, '47). Thus, an interrelationship exists among vitamin B_{12}, folacin and choline or methionine.

The results reported in the present paper show that for maximum chick growth both vitamin B_{12} and folacin are required. When both are supplied in the diet, the resulting growth increment is an additive one (fig. 1). In short-term experiments either folacin or vitamin B_{12} had a pronounced effect on feathering; however, folacin was superior to vitamin B_{12}. Of the chicks maintained for 20 weeks or longer, those receiving the basal diet plus both folacin and vitamin B_{12} and 0.10% or 0.60% choline chloride had superior feather development as compared to those receiving either a folacin or a vitamin B_{12} supplement.

Folacin and vitamin B_{12} appeared capable of replacing each other for normal hemoglobin production under the experimental conditions reported. The omission of both from the diet did not consistently produce a severe anemia. The average hemoglobin level of 45 birds receiving the basal diet plus 0.60% choline chloride was 7.5 gm %. The addition of both vitamins consistently produced slightly higher hemoglobin values than did either one of the two.

The highest incidence of perosis (80%) was noted in the chicks receiving the basal diet plus 0.10% choline chloride, and the highest mortality (54%) and a spastic cervical paralysis occurred in the groups receiving the basal diet plus 0.60% choline chloride. Although vitamin B_{12} greatly reduced the incidence of perosis and mortality, it did not give complete protection. Only an occasional case of perosis occurred in the chicks fed 0.60% choline chloride plus 2 mg folacin per

kilogram of diet, whereas a 66% incidence of perosis resulted when the supplementary choline level was reduced to 0.10%. When extremely high levels of folacin (100 mg per kilogram) were fed without a supplementary source of vitamin B_{12}, perosis did not occur even at the 0.10% choline level. Daniel and co-workers ('46) observed perosis in chicks on a purified diet without folacin and increased the incidence by adding sulfasuxidine. The higher incidence of perosis on the sulfa drug-containing diet and the higher requirement of folacin for protection led to their suggestion that folacin stimulated the intestinal flora to produce an unknown antiperotic factor. The data shown in table 3 indicate that this unknown factor could be vitamin B_{12}. In the presence of vitamin B_{12} (150 μg per kilogram of diet) and 0.10% choline chloride, 0.20 mg per kilogram of folacin was adequate to protect against perosis. Supplementing the basal diet with folacin, vitamin B_{12} and 1.31% DL-methionine resulted in a 100% incidence of perosis. This emphasizes that the role of choline in the prevention of perosis cannot be filled by methionine, vitamin B_{12} or folacin. The results of these experiments indicate that an interrelationship exists among folacin, vitamin B_{12} and choline in protecting against perosis.

Keith et al. ('48) reported that the ratios of chick liver weights to body weights were inversely related to the folacin content of a purified diet and that the gallbladders of the deficient chicks were greatly enlarged. Similar observations were made in the studies herein reported. For normal liver weight to body weight ratio and gallbladder size both folacin and vitamin B_{12} appeared to be essential.

The studies presently reported suggest that in the nutrition of the chick and the rat vitamin B_{12} exerts its maximum effect in the presence of folacin, and demonstrate that choline, vitamin B_{12} and folacin are interrelated in their physiological functions. Various types of megaloblastic anemias in humans have responded to folacin (reviewed by Jukes and Stokstad, '48) or vitamin B_{12} (West, '48; Spies and co-workers, '49) or

choline chloride (Moosnick and co-workers, '45; Davis and Brown, '47). The present results suggest that in the treatment of megaloblastic anemias it may be advantageous to consider the use of all three of these nutrients.

SUMMARY

1. The incidence and severity of renal injury in weanling rats fed diets low in choline and methionine were decreased by supplementing the diet with folacin. When both folacin and vitamin B_{12} were added to the basal diet supplemented with 0.04% choline chloride or 0.128% DL-methionine, there was complete protection against kidney damage.

2. Both folacin and vitamin B_{12} are required for maximum chick growth. When both are present in the diet, the resulting gain is an additive effect.

3. Under the experimental conditions reported in this paper vitamin B_{12} could replace folacin for hemoglobin production in chicks; however, slightly higher hemoglobin levels were consistently observed when both nutrients were present in the diet.

4. Both vitamin B_{12} and folacin in addition to choline are concerned in the prevention of perosis in the chick. A folacin deficiency produced a much higher incidence of perosis than a vitamin B_{12} deficiency.

5. The folacin requirement of chicks under the experimental conditions reported was 0.2 mg per kilogram of diet or less when the basal diet was supplemented with 150 μg vitamin B_{12} per kilogram and 0.10% choline chloride. The vitamin B_{12} requirement was more than 5 μg per kilogram even when an extremely high level of folacin (100 mg per kilogram) was fed.

6. The nutritional requirements of the chick and rat for folacin, vitamin B_{12} and choline are interrelated, and a specific requirement for one of these nutrients cannot be established unless the level of other two is taken into consideration.

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PLATE 1

EXPLANATION OF FIGURES

2 Chicks 6 weeks old (exp. 8). The one on the left received 0.60% choline chloride but no folacin or vitamin B_{12} (165 gm body wt.). The one on the right received 0.60% choline chloride plus 30 μg crystalline vitamin B_{12} per kilogram diet (494 gm body wt.). Note the perosis in this chick.

3 The liver on the left is from the chick on the left and that on the right is from the chick on the right in figure 2. Note the enlarged gallbladder from chick receiving no folacin or vitamin B_{12}.

4 Chicks 6 weeks old (exp. 8). Chick on the left was fed 0.60% choline chloride plus 2 mg folacin and 30 μg vitamin B_{12} per kilogram of diet (640 gm body wt.). Chick on the right was fed 0.60% choline chloride plus 2 mg folacin per kilogram diet (500 gm body wt.).

5 Chicks 20 weeks old (exp. 3). Chick on the left received 0.60% choline chloride plus 2 mg folacin per kilogram diet. Chick on the right was fed only the basal diet plus 0.60% choline chloride.

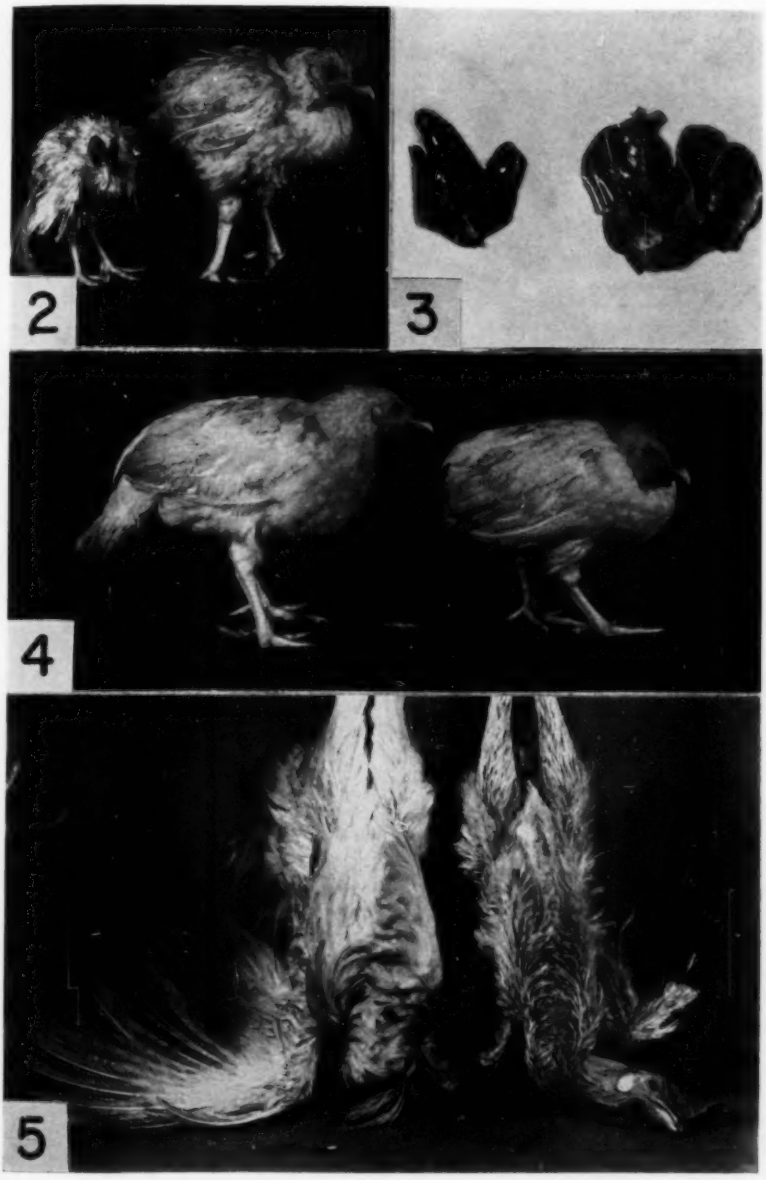
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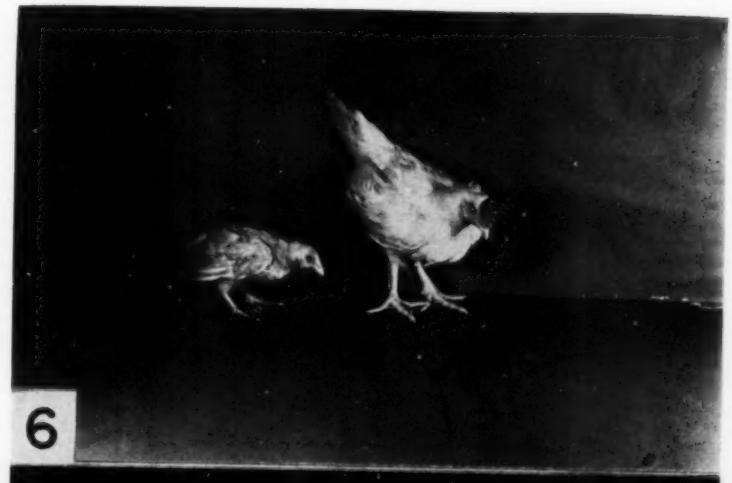
PLATE 2

EXPLANATION OF FIGURES

6 Chicks 6 weeks old (exp. 10). Chick on the left received 0.10% choline chloride but no folacin nor vitamin B_{12}. Chick on the right received 0.10% choline chloride plus folacin plus vitamin B_{12}.

7 Chicks 6 weeks old (exp. 10). Chick on the left received 0.10% choline choride, 0.2 mg folacin and 150 μg vitamin B_{12} per kilogram diet. Chick in the center received the same diet, but the folacin was increased to 2.0 mg per kilogram. Chick on the right received 0.60% choline chloride, 2.0 mg folacin and 30 μg vitamin B_{12} per kilogram diet.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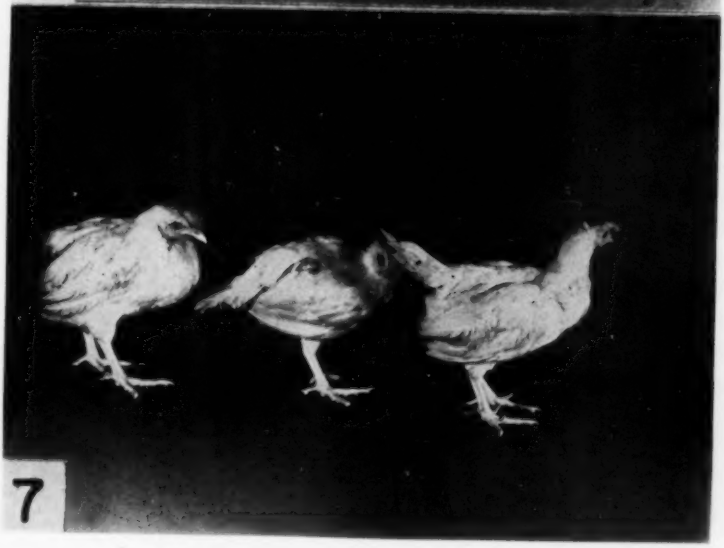
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THE RELATION OF THE STRUCTURE OF CHOLINE-LIKE COMPOUNDS TO RENAL ANTIHEMORRHAGIC ACTION[1]

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The experiments described in this communication were conducted from 1939 to 1943 as part of a study of the relation between chemical structure and the various biological actions of choline. Some of the findings were made available to Dr. T. H. Jukes, who presented them in a symposium of the American Institute of Nutrition in 1941; also, many were recorded, as personal communications, in papers by Moyer and duVigneaud ('42) and Jukes ('47).

The recent studies by Borsook and Dubnoff ('47), by Dubnoff and Borsook ('48a, b), and by Dubnoff ('49) of the enzymatic reactions involved in transmethylation have indicated the desirability of a more detailed presentation of our earlier findings. The methyl-donating property of the sulfur analogue of betaine, dimethylthetin, $(CH_3)_2S^+ \cdot CH_2COO^-$, demonstrated by the work of Dubnoff and Borsook ('48a) and of duVigneaud, Moyer and Chandler ('48), was suggested by our finding in 1939 of the lipotropic activity of this compound, and this was noted in the paper by Moyer and duVigneaud in 1942. The concept of the biologically labile

[1] These studies were begun in the School of Medicine, Washington University, St. Louis, in the department of Professor C. F. Cori, and were supported, in part, by a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association; the final experiments were done in the Medical Research Division of Sharp and Dohme, Inc., Glenolden, Pennsylvania.

methyl group had not been formulated at the time our first observations were made, and the metabolic functions of choline and related compounds were being investigated through the use of arsenic, phosphorus, and sulfur analogues. Since the phosphorus and arsenic analogues of choline are markedly lipotropic, while the corresponding analogues of betaine are inactive, it had been concluded that the lipotropic action of choline involves the utilization of the intact molecule, and that the aerobic conversion of choline to betaine which occurs in the liver and kidney of the rat and certain other species is not requisite to this function of choline (Welch, '36; Welch and Welch, '38a). This conclusion was supported by the demonstration that the arsenic analogue of choline, used as a labeled molecule prior to the availability of suitable isotopes as tracers, is incorporated into lecithin and sphingosinephosphorylcholine (Welch, '36; Welch and Landau, '42); but, as was shown later, it does not serve as a donor of a methyl group (duVigneaud, et al., '40; Welch, '41; Moyer and duVigneaud, '42; Almquist and Jukes, '42).

EXPERIMENTAL

The present studies were carried out with weanling rats of 23 to 29 days of age; these were placed in wire-mesh cages and were given water and food ad libitum. In the experiments numbered 1 to 10 the rats used were bred in the laboratory; in the subsequent experiments they were obtained from a commercial source.[2]

The diets varied slightly in composition, but all were high in fat, 40%, and contained carbohydrate, 38%, and protein (alcohol-extracted casein), 15%, supplemented with L-cystine, 0.5%.

In experiments 1 to 12 the diet contained beef fat and corn oil, each 16%, commercial hydrogenated cottonseed oil,[3] 7.9%, and percomorph oil, 0.1%; in experiments 13 and 14, hydrogenated cottonseed oil,[4] 38%, and corn oil, 2%,

[3] Sunny Hill.
[3] Crisco.
[4] Primex.

were used, together with a vitamin concentrate supplying, per 100 gm of diet: A, 5,000 units; D, 1,000 units; and mixed tocopherols, 3 mg. In experiments 1 to 12 the diet contained sucrose, 16%, and dextrin, 32%; in experiments 13 and 14, dextrose, 26%, and dextrin, 12%, were used. Salts, 4%, were supplied by mixture no. 2 of the U.S.P.X. and bulk was provided by purified agar, 2%. Although the duration of the experiments was never more than 12 days, attention was given to the vitamin content of the rations. In the earlier experiments (1 to 10) vitamins were supplied by a rice polishings extract,[5] 0.5%, supplemented (in experiments 3 to 10) with thiamine hydrochloride, riboflavin and nicotinic acid, each 0.0005%. In experiments 11 and 12 synthetic vitamins were used at the following percentages: thiamine hydrochloride, riboflavin and pyridoxine hydrochloride, each 0.003; nicotinic acid, 0.025; calcium pantothenate, 0.02. In experiments 13 and 14 the vitamin mixture supplied the following percentages: thiamine hydrochloride and pyridoxine hydrochloride, each 0.001; riboflavin, 0.002; nicotinic acid and pantothenic acid, each 0.02; 2-methyl-1, 4-naphthohydroquinone diacetate, 0.0002.

Detailed information concerning the synthesis of the various compounds used does not appear to be essential. Well-known methods of methylation or ethylation were employed, such as were described earlier by Jukes and Welch ('42), or trimethylamine or its appropriate analogue, either prepared by standard methods or purchased, was condensed with either ethylenebromohydrin or bromoacetic acid. After conversion of the resultant salt to the corresponding chloride by treatment with silver chloride, the compound was isolated and recrystallized from ethanol with the aid of absolute ether. Adequate purity was insured by nitrogen, phosphorus, arsenic and halogen determinations. Sulfurylcholine and phosphoryl-β-methylcholine were prepared from choline or its β-methyl derivative by esterification with the appropriate acid.[6]

[5] Ryzamin B, prepared by the Burroughs Wellcome Company, Tuckahoe, New York.

[6] Serine betaine was a gift from Dr. Herbert Carter of the University of Illinois, and β-methylcholine chloride was generously supplied by Merck and Co., Inc. Arsenocholine chloride was a gift from Hoffmann-La Roche, Inc.; the arsenobetaine hydrochloride and the corresponding phosphorus analogues were prepared by the author. In the later experiments (12 to 14) betaine aldehyde chloride and the ethyl homologues of choline chloride were prepared by Dr. J. M. Sprague and his associates of Sharp and Dohme, Inc., to whom our thanks are due.

Accurate estimation of the antihemorrhagic potency of compounds is complicated by the lack of entirely adequate criteria. In the tables which follow, the degree of renal pathologic change is indicated both on the basis of the average weight and the range in weight of the paired kidneys (engorgement with blood causes a marked increase in renal weight), and on a scoring system based on the gross appearance of the kidneys at autopsy; the latter method appears to be of much greater usefulness in assessing activity. The scoring of renal hemorrhagic changes was based on the following system: 0, no evidence of hemorrhage; 1.0, slightly involved; 2.0, moderately involved; 3.0, severely involved; 4.0, massive engorgement with blood. In the tables the average score is presented, together with the percentage of kidneys showing damage of any degree. As a rule the damage to each of the two kidneys was of very similar intensity; when differences were seen, the situation encountered in the more severely involved kidney was recorded. It should be noted that all compounds were administered at levels equivalent on a molecular weight basis to a stated level of choline chloride. Although determinations of the total lipid content of the liver were made in all experiments (except 11), the analytical data have not been presented in the tables except in table 1, where the effect of varying doses of choline chloride is shown. Under the conditions of these experiments of short duration, the fat content of the liver was influenced erratically by compounds with lipotropic activity. Even in the absence of added lipotropic agents, widely variable values for total liver fat were obtained; thus, the average hepatic fat content of 88 unsupplemented rats, 18.6% (1.28 gm of liver fat per 100 gm of body weight), has little significance and was omitted from table 1. Within individual experiments it was possible, in some cases, qualitatively to detect marked lipotropic activity, and mention of such activity will be made in the discussion of the findings. Liver fat was determined by the method described in an earlier publication (Welch and Welch, '38b).

The results of the various experiments are summarized in tables 1 to 4, in which, to avoid the complexities of chronological presentation, the findings obtained with each compound have been collected together. In each case, however, the number of the experiment from which the results were derived has been indicated, so that comparison of results within a single experiment is made possible. Such a comparison does not appear to modify the conclusions in any significant manner.

TABLE 1

Renal antihemorrhagic and lipotropic activity of choline chloride in weanling rats

EXP'T. NO.	DIETARY LEVEL OF CHOLINE CHLORIDE	NO. OF RATS	AVE. INCREASE IN WT. OF RATS	KIDNEYS			LIVER	
				Ave. wt. and range	Per cent damaged	Ave. score	Per cent fat	Liver fat per 100 gm body weight
	mg/gm of diet		*gm*	*gm*				*gm*
..	0	105	9.9	0.90 (0.50–1.36)	93	3.2	...	...
13	0.25	10	11.6	0.77 (0.42–1.10)	70	2.8	27.1	2.19
14	0.38	10	23.9	0.71 (0.56–1.10)	60	1.2	27.1	2.14
12	0.5	10	21.6	0.79 (0.67–0.88)	30	0.3	10.6	0.60
14	0.75	10	20.5	0.56 (0.48–0.72)	0	0.0	17.6	1.05
13	1.0	8	18.2	0.52 (0.51–0.56)	0	0.0	16.5	0.95
12	2.0	10	22.4	0.77 (0.68–0.89)	0	0.0	3.4	0.20
1	2.5	11	10.1	0.48 (0.38–0.59)	0	0.0	3.2	0.18

RESULTS AND DISCUSSION

Many advances in our knowledge of the processes involved in the transfer of the methyl group have occurred since these studies were made over 6 years ago. Accordingly, it would be somewhat inappropriate to present an exhaustive interpretation of these findings in the light of this newer knowledge. However, attention will be drawn to the results obtained with some of the compounds and the significance of these results will be reviewed briefly.

It will be noted (table 1) that the combined control groups, given only the basal diet and containing a total of 105 animals, had paired kidneys which varied in weight from 0.50 to

1.36 gm, with an average weight of 0.90 gm per pair. Of these kidneys, over 93% showed evidence of hemorrhagic injury and the average "score" was 3.2. The average weight of 116 pairs of normal kidneys obtained from rats fed completely protective amounts of choline chloride (or another very effective compound) was 0.54 gm. Table 1 shows that the renal hemorrhages characteristically produced by the basal diets used were prevented by choline chloride at a level of about 0.75 mg per gram. However, only slight protection was afforded by 0.25 mg of choline chloride per gram of diet, and the level that would protect 50% of the animals may be estimated to approximate 0.4 mg per gram of diet (0.04%). It is also evident that a much higher dietary level of choline chloride is necessary to prevent accumulation of fat in the liver than is required for the complete protection of the kidneys.

In table 2 it will be seen that introduction into the choline molecule of ethyl groups in place of two of the three methyl groups results in a compound that is markedly active antihemorrhagically; in fact the limited data available suggest that the compound may be as active as choline itself. The corresponding dimethyl-monoethyl compound was found to possess a similar order of activity. Although the higher activity of the mono- and di-ethyl compounds, in comparison with the triethyl homologue (see below), might be attributed to their serving also as donors of a methyl group, the mono-methyl compound did not appear to permit homocystine to be utilized for growth in two rats described by Welch ('41), nor did it replace choline in promoting growth in the chick, although in that species it is a potent antiperotic agent (Jukes and Welch, '42). It is possible that this compound, due to closer structural resemblance to choline, functions more effectively than the triethyl homologue in the synthesis of phospholipids. At the levels fed, definite evidence of toxic effects was not produced by the three ethyl-containing

TABLE 2

Renal antihemorrhagic activity of compounds related to choline chloride

COMPOUND TESTED, AND FORMULA	EXPERIMENT NO.	DOSAGE AS CHOLINE CHLORIDE EQUIVALENT	NO. OF RATS	AVERAGE INCREASE IN WEIGHT OF RATS	KIDNEYS		
					Average weight and range	Per cent damaged	Average score
		mg/gm of diet		*gm*	*gm*		
Dimethylethyl-β-hydroxyethylammonium chloride: $(CH_3)_2(C_2H_5)N\cdot CH_2CH_2OH(Cl)$	14	1.0	10	26.9	0.61 (0.50–0.76)	0	0.0
	11	4.0	15	12.7	0.57	0	0.0
Methyldiethyl-β-hydroxyethylammonium chloride: $CH_3(C_2H_5)_2N\cdot CH_2CH_2OH(Cl)$	14	1.0	10	13.0	0.51 (0.46–0.60)	0	0.0
	11	4.0	15	4.4	0.49	0	0.0
Triethyl-β-hydroxyethylammonium chloride: $(C_2H_5)_3N\cdot CH_2CH_2OH(Cl)$	14	1.0	10	8.1	0.68 (0.52–0.90)	50	1.9
	2	2.0	3	9.4	0.47 (0.43–0.50)	0	0.0
	9	4.0	6	1.8	0.39 (0.30–0.48)	0	0.0
Tripropyl-β-hydroxyethylammonium chloride: $(C_3H_7)_3N\cdot CH_2CH_2OH(Cl)$	3	4.0	5	8.0	0.71 (0.50–0.91)	100	3.0
	9	4.0	6	9.4	0.64 (0.48–0.82)	67	1.5
Arsenocholine (Trimethyl-β-hydroxyethylarsonium) chloride: $(CH_3)_3As\cdot CH_2CH_2OH(Cl)$	14	1.0	10	21.9	0.62 (0.50–0.92)[1]	20	0.5
	4	2.5	6	15.8	0.56 (0.49–0.59)	0	0.0
Phosphocholine (Trimethyl-β-hydroxyethylphosphonium) chloride: $(CH_3)_3P\cdot CH_2CH_2OH(Cl)$	9	4.0	6	10.6	0.49 (0.42–0.55)	0	0.0
β-Methylcholine chloride: $(CH_3)_3N\cdot CH_2CH(CH_3)OH(Cl)$	5	2.5	5	8.7	0.62 (0.50–0.86)	100	2.0
	4	2.5	6	15.9	0.62 (0.54–0.70)	67	0.7
	12	4.0	10	18.3	0.71 (0.47–0.96)	40	0.6
	12	10.0	10	19.3	0.76 (0.62–0.99)	40	0.4
	13	10.0	10	9.4	0.41 (0.34–0.44)	0	0.0
	13	20.0	10	10.0	0.41 (0.39–0.44)	0	0.0
Aminoethanol: $H_2N\cdot CH_2CH_2OH$	14	20.0	10	3.5	0.96 (0.66–1.22)	100	4.0
Dimethylaminoethanol: $(CH_3)_2N\cdot CH_2CH_2OH$	14	10.0	10	24.5	0.63 (0.50–0.72)	0	0.0

[1] With the exception of two pairs of kidneys (weight, 0.52 gm and score, 1.0; weight, 0.92 gm and score, 4.0, respectively), all kidneys appeared normal (average weight, 0.59 gm).

homologues, except that the gain in weight of the rats during the experimental period was diminished as the number of ethyl groups was increased.

The data concerned with the triethyl homologue of choline chloride demonstrate the definite renal antihemorrhagic action of this compound that is unable to serve as a methyl donor. On a molar basis, the triethyl homologue appears to have about one-half the activity of choline chloride. Proof of the utilization of this substance for the synthesis of phospholipids has been supplied by McArthur ('46), who isolated the compound from hepatic lecithins of rats fed the homologue. Earlier, Channon and Smith ('36) had noted the lipotropic activity of the compound, but Channon, Platt, Loach and Smith ('37), on the basis of their failure to accomplish the isolation of the homologue from the phospholipids of rats fed the triethyl compound, had erroneously questioned the finding by Welch ('36) of arsenocholine in phospholipids of rats fed the arsenic analogue of choline chloride. Determination of liver lipids in the experiments presently reported showed that the compound also exerted a significant lipotropic action at dietary levels of 0.2% or higher.

The corresponding tripropyl homologue has very little activity as a renal antihemorrhagic agent. At the level fed, it exerted no lipotropic action.

Studies of the arsenic analogue of choline chloride (table 2) indicate that this compound, which, as has been mentioned earlier, appears not to serve as a methyl donor, is very effective in preventing renal hemorrhages. As was reported for its lipotropic activity in mice (Welch and Welch, '38a), the renal antihemorrhagic potency of the compound, on a molar basis, appears to be about one-half that of choline chloride. The phosphorus analogue tested at a level equivalent to 4 mg of choline chloride per gram of diet, was found to be a potent renal antihemorrhagic agent; at this level the compound also exerted a very significant lipotropic action.

β-Methylcholine chloride was found to possess relatively little renal antihemorrhagic activity, an observation which

was anticipated in view of the report by Welch and Welch ('38a) of its apparent lipotropic inactivity in mice. Subsequently, evidence that it does not serve as a methyl donor was presented (Welch, '41), and Jukes and Welch ('42) found it to exert only a weak antiperotic action in chicks. Since there were no obvious reasons why this compound should not be utilized in lieu of choline for the synthesis of phosphorylated complexes, it was deemed important thoroughly to test the compound as a renal antihemorrhagic agent. Accordingly it was fed at 4 levels (choline equivalent: 2.5 to 20 mg per gram of diet) in 6 experiments (table 2). The results show clearly that β-methylcholine chloride is only weakly active as a renal antihemorrhagic agent, and suggest that the compound has about one-tenth to one-twentieth the activity of choline chloride. Only at the very high dietary level of 2% was evidence suggestive of a slight lipotropic activity disclosed.

In the synthesis of phospholipids, phosphorylation of choline has not been shown to be the first step. However, as a test of the possibility that the poor activity of β-methylcholine might be due to a relative insusceptibility to esterification, the phosphate ester was prepared and was administered subcutaneously as the sodium salt. It was appreciated, of course, that contact with phosphatases was not prohibited and that the ester might be converted rapidly to β-methylcholine; the toxic effects of dosages larger than that used, equivalent to 25 mg of choline chloride daily, suggest that hydrolysis probably occurs fairly rapidly following injection of the ester. The subcutaneous administration of the ester, in the dosage described, prevented the development of hemorrhagic kidneys in 4 of 6 animals (score: 1.0). However, these findings offer no real support to the hypothesis that the injected phosphorylated compound is more active than orally administered β-methylcholine chloride. Accordingly, the reasons for the remarkably low potency of β-methylcholine as a lipotropic, antiperotic and renal antihemorrhagic agent are still to be discovered. In view of recent findings (Dubnoff,

'49; Muntz, '50) indicating that in order to serve as a methyl donor choline must first be oxidized to betaine, it is not surprising that β-methylcholine should be inactive as a source of labile methyl, since it is not a primary alcohol and oxidation could not form a carboxyl group.

Aminoethanol, at a level equivalent to 20 mg of choline chloride per gram of diet, was completely ineffective, whereas dimethylaminoethanol, at half that level, was fully protective to the kidneys (table 2) and also was markedly lipotropic. Apparently, the labile methyl supply, although adequate for the formation of an effective amount of choline from dimethylaminoethanol, is grossly inadequate for the three-step synthesis from aminoethanol, which Stetten ('41) has shown to be accomplished by the rat. Possibly, with the very high level of aminoethanol fed in this experiment, available methyl groups are used primarily for the formation at monomethylaminoethanol, and to a lesser extent for the formation at dimethylaminoethanol, so that little or no choline can be formed under these conditions.

The renal antihemorrhagic inactivity of the sulfuric acid ester of choline is of some interest, because this compound was shown by Woolley and Peterson ('37) to occur in nature. At a level equivalent to 4 mg of choline chloride per gram of diet, the kidneys of 7 rats were all severely damaged (score: 3.7). It appears that in the rat choline cannot be released from this remarkably stable ester. *i*-Inositol also was tested because of its known participation in the formation of certain lipid complexes. However, as has been reported by Handler ('46), it appeared to exert no protective action; at a level of 1% in the diet, each of 9 rats developed renal injury (score: 2.9).

Betaine aldehyde is produced enzymatically by the oxidation of choline by the rat liver *in vitro* (Mann and Quastel, '37). Presumably its renal antihemorrhagic and other activities depend on its subsequent oxidation to betaine, which then can supply a single methyl group for choline synthesis. However, there are no data that exclude the possibility of

TABLE 3

Renal antihemorrhagic activity of betaine hydrochloride and related compounds

COMPOUND TESTED, AND FORMULA	EXPERIMENT NO.	DOSAGE AS CHOLINE CHLORIDE EQUIVALENT	NO. OF RATS	AVERAGE INCREASE IN WEIGHT OF RATS	KIDNEYS		
					Average weight and range	Per cent damaged	Average score
		mg/gm of diet		*gm*	*gm*		
Betaine aldehyde chloride: $(CH_3)_3N \cdot CH_2CHO(Cl)$	12	2.0	10	23.8	0.76 (0.63–0.89)	40	0.5
	12	4.0	10	24.7	0.68 (0.56–0.82)	0	0.0
Betaine hydrochloride: $(CH_3)_3N \cdot CH_2COOH(Cl)$	14	1.0	10	16.9	0.87 (0.50–1.36)	70	2.7
	8	5.0	6	8.1	0.47 (0.53–0.61)	0	0.0
Phosphobetaine hydrochloride: $(CH_3)_3P \cdot CH_2COOH(Cl)$	10	4.0	6	5.8	0.88 (0.63–1.10)	100	3.5
Arsenobetaine hydrochloride: $(CH_3)_3As \cdot CH_2COOH(Cl)$	5	2.5	5	7.4	0.70 (0.45–0.90)	80	2.6
	9	4.0	6	9.5	0.86 (0.68–1.17)	100	3.3
α-Alanine betaine (α-Methylbetaine) hydrochloride: $(CH_3)_3N \cdot CH(CH_3) \cdot COOH(Cl)$	6	4.0	8	12.0	0.56 (0.49–0.64)	50	0.5
	10	4.0	7	6.7	0.58 (0.41–0.71)	57	1.1
	11	4.0	9	10.8	0.82 (0.57–1.07)	67	2.4
	12	4.0	9	14.7	0.98 (0.78–1.38)	100	3.2
	12	10.0	8	17.5	0.78 (0.58–1.13)	63	1.4
	13	20.0	9	8.4	0.86 (0.52–1.40)	100	3.4
Serine betaine hydrochloride $(CH_3)_3N \cdot CH(CH_2OH)COOH(Cl)$	8	5.0	5	5.4	0.92 (0.58–1.22)	100	3.0

its direct participation in transmethylation processes. The present findings (table 3) indicate that it is significantly less active than choline chloride (perhaps about one-fourth as active), and of about the same order of activity as betaine hydrochloride.

Betaine hydrochloride was tested in two experiments; the findings are in substantial agreement with those of Griffith and Mulford ('41), who considered the compound to be about one-third as active as choline chloride in the prevention of the hemorrhagic kidney syndrome, and made the shrewd suggestion that only one of the three methyl groups of betaine is labile.

In keeping with the concept that choline, arsenocholine and phosphocholine act lipotropically as intact molecules (Welch and Welch, '38a), the findings presented in table 3 indicate that the arsenic and phosphorus analogues of betaine are without significant renal antihemorrhagic activity.

The betaine corresponding to α-alanine (sometimes termed α-methyl-betaine) has been reported to have lipotropic activity in mice (Welch and Welch, '38a). However, activity as a methyl donor was not observed by Moyer and duVigneaud ('42). Accordingly, a number of tests of this compound for renal antihemorrhagic activity were made, at first with material prepared by us by methylation with methyl iodide (experiments 6, 7 and 11); later with material kindly supplied by Dr. duVigneaud and prepared by methylation with dimethylsulfate (experiment 12); and finally with material kindly prepared by Mr. C. Zeigler and Dr. J. M. Sprague, of Sharp and Dohme, Inc., by condensation of trimethylamine and α-bromopropionic acid (experiment 13). Examination of the findings recorded in table 3 indicates that variable evidence of slight activity was obtained with α-alanine betaine prepared by methylation of alanine, particularly that prepared by treatment with methyl iodide. Accordingly, material of high purity (theory: N, 8.36%; Cl, 21.15%; found: N,8.12, 8.10%; Cl, 21.10, 21.07%), prepared by Zeigler and Sprague by an entirely different method, was then tested (experiment

13) at twice the highest level used previously, and no evidence of either renal antihemorrhagic (table 3) or lipotropic activity was found. It is conceivable that during the methylation of alanine small amounts of an active compound were formed whose properties and composition led to its undisclosed contamination of batches prepared in this way. Certainly the results obtained with the last-described material indicate that α-alanine betaine, prepared by the condensation procedure, is quite inactive at a dietary level of 2%.

Another closely related betaine, that of serine, exerted no activity at a level equivalent to 5 mg of choline chloride per gram of diet (table 3). It would appear that these two α-substituted derivatives of betaine are insusceptible to attack by the enzyme system studied by Dubnoff and Borsook ('48a) and therefore are unable to release a methyl group for methionine and choline synthesis.

Of the greatest interest was the finding that the sulfur analogue of betaine $(CH_3)_2S^+ \cdot CH_2COO^-$, usually termed dimethylthetin, is a potent lipotropic and renal antihemorrhagic agent. As mentioned earlier, this compound originally was found by us to be lipotropically active (see Moyer and duVigneaud, '42) and extensive studies have been made by duVigneaud, Moyer and Chandler ('48) and by Dubnoff and Borsook ('48a) of its methyl-donating property. In these studies the compound was used as the hydrochloride, at dietary levels molecularly equivalent to choline chloride concentrations of from 1 to 4 mg per gram of diet (table 4). The levels of dimethylthetin required partially to protect the kidneys show some variation. It is assumed that the somewhat different conditions in the various experiments altered slightly the ability of the animals to transfer a methyl group from the sulfur analogue of betaine to various compounds which occur in the diet or are produced metabolically, and which serve as precursors of choline.

Replacement of one of the two methyl groups of dimethylthetin with an ethyl group (table 4) did not abolish renal protective activity, but exclusion of the possibility of methyl-

TABLE 4

Renal antihemorrhagic action of dimethylthetin and related compounds

COMPOUND TESTED, AND FORMULA	EXPERIMENT NO.	DOSAGE AS CHOLINE CHLORIDE EQUIVALENT	NO. OF RATS	AVERAGE INCREASE IN WEIGHT OF RATS	KIDNEYS		
					Average weight and range	Per cent damaged	Average score
		mg/gm of diet		*gm*	*gm*		
Dimethylthetin hydrochloride: $(CH_3)_2S \cdot CH_2COOH(Cl)$	12	1.0	9	26.2	0.90 (0.70–1.13)	44	1.0
	13	2.0	8	15.8	0.59 (0.50–1.04)[1]	13	0.5
	10	3.0[2]	7	11.1	0.52 (0.33–0.81)	29	0.7
	11	4.0	15	14.3	0.57	0	0.0
	14	4.0	10	22.0	0.58 (0.48–0.70)	0	0.0
Methylethylthetin hydrochloride: $CH_3(C_2H_5)S \cdot CH_2COOH(Cl)$	12	4.0	10	18.0	0.72 (0.62–0.89)	40	0.2
	14	4.0	10	24.0	0.70 (0.58–1.00)[3]	10	0.4
Diethylthetin hydrochloride: $(C_2H_5)_2S \cdot CH_2COOH(Cl)$	12	4.0	10	10.3	0.99 (0.69–1.27)	80	3.0
	13	10.0	7	0	0.74 (0.64–0.90)	100	2.9

[1] With the exception of one pair of kidneys (weight, 1.04 gm; score, 4.0), all kidneys appeared normal (average weight, 0.52 gm).

[2] During the first 4 days the level in the diet was 4 mg per gram; the reduction in the amount was due to a shortage in the supply.

[3] With the exception of one pair of kidneys (weight, 1.0 gm; score, 4.0), all kidneys appeared normal (average weight, 0.66 gm).

donation, by replacing the remaining methyl group with a second ethyl group, caused a disappearance of such activity. Similar results have been obtained by Maw and duVigneaud ('48b), who stated that the activity of dimethylthetin cannot be attributed to its being the sulfur analogue of betaine, because the sulfur analogue of choline proved to be completely ineffective as a methyl donor (Maw and duVigneaud, '48a). However, the findings of Dubnoff ('49) suggest that the enzymic conversion of choline to betaine enables it to serve as a methyl donor for homocysteine methylation, and it would appear that this enzyme system is unable effectively to oxidize sulfocholine to sulfobetaine (dimethylthetin). Actually, the activity of dimethylthetin, coupled with the inactivity of sulfocholine, is very strong support for the concept that betaine and not choline is the true donor of the methyl group. The recent observation of Muntz ('50) that N^{15}-choline is converted anaerobically by rat liver homogenates, not to N^{15}-dimethylaminoethanol but to N^{15}-dimethylglycine, leaves no doubt that under these conditions choline is oxidized and the labile methyl group is derived from betaine rather than from its precursor, choline.

The demonstration by Dubnoff and Borsook ('48a) of the existence of an enzyme system capable of transferring methyl groups from dimethylthetin to homocysteine suggests that the sulfur analogue of betaine may possibly play a physiological role in transmethylation mechanisms. The related compound, $(CH_3)_2S^+ \cdot CH_2CH_2COO^-$, occurs naturally (Challenger and Simpson, '47) and has been shown (Maw and duVigneaud, '48b) to be capable of serving as a methyl donor.

The findings presented herein are in agreement with our earlier conclusions (Welch and Welch, '38a) concerning the lipotropic action of choline, and indicate further that the renal antihemorrhagic action of this compound, originally observed by Griffith and Wade ('39a, b) in weanling rats deficient in choline, is also due to a mechanism involving the intact molecule of choline. Presumably this function is related to the synthesis of essential conjugated forms of choline, such

as the lecithins and the derivatives of sphingosinephosphorylcholine (Welch, '36; Welch and Landau, '42). That the transfer of the methyl group is not essential to renal protection is indicated by the high activity of the triethyl homologue of choline as well as the arsenic analogue of choline, neither of which appears to serve directly or indirectly as a methyl donor. The renal antihemorrhagic activity of the sulfur analogue of betaine, dimethylthetin, and its homologue, methyl-ethylthetin, coupled with the inactivity of diethylthetin, suggests that the two methyl-containing thetins, like betaine itself, exert their renal antihemorrhagic action by contributing a methyl group which is used in the synthesis of choline, a substance critically involved in the development of essential tissues of the kidney.

SUMMARY

The ability of choline, betaine, dimethylthetin, and various related compounds to protect the weanling rat from the renal hemorrhagic syndrome of choline deficiency has been studied. The frequency of hemorrhagic change in the kidney is correlated better with the dietary level of choline than is the average weight of the paired kidneys. Under the conditions described, the dietary level of choline chloride required to protect 50% of the animals on experiment is about 0.04%, while 0.075% affords protection to all animals. It is noted that the kidney is protected by a lower dietary level of choline than is required for preventing the deposition of fat in the liver.

The marked renal protective action of the triethyl homologue and of the arsenic analogue of choline chloride are demonstrated. This activity, coupled with the inability of these compounds to serve as donors of a methyl group, permits the conclusion that choline acts by satisfying a requirement of the growing kidney for the intact molecule of choline and not primarily for the labile methyl group of which choline, by virtue of its conversion to betaine, is a donor. The phos-

phorus analogue of choline chloride is also a potent renal protective agent. In keeping with earlier findings concerning the lipotropic inactivity of the arsenic and phosphorus analogues of betaine hydrochloride, these compounds are shown to exert no renal antihemorrhagic effect.

The monoethyl and diethyl homologues of choline chloride are very effective antihemorrhagically, but the tripropyl homologue is inactive.

β-Methylcholine chloride is about one-tenth to one-twentieth as active as choline chloride in preventing the renal hemorrhages of choline deficiency. The activity of the phosphoric acid ester of this derivative of choline, administered subcutaneously, probably is equivalent to its β-methylcholine content.

Betaine aldehyde chloride and betaine hydrochloride are active renal antihemorrhagic (and lipotropic) agents, but the activity appears to approximate one-fourth that of choline chloride. Although some samples of α-alanine betaine appeared to protect the kidneys to a slight extent, it is concluded that the pure compound is inactive in this respect as well as lipotropically. The betaine of serine is also inactive.

Dimethylthetin (the sulfur analogue of betaine) and methylethylthetin are potent renal antihemorrhagic agents, while diethylthetin is inactive. The ability of the methyl-containing thetins, like betaine, to donate a methyl group for the synthesis of choline from precursors which occur in the diet or are produced metabolically is discussed.

Inactive as renal antihemorrhagic agents are the sulfuric acid ester of choline, *i*-inositol, and aminoethanol. The activity of dimethylaminoethanol is noted and its mechanism of action discussed.

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FURTHER STUDIES ON THE RELATIONSHIP BETWEEN ILLUMINATION AND THE ASCORBIC ACID CONTENT OF TOMATO FRUITS

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ONE FIGURE

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For a number of years it has been recognized that the amount of light is an important factor determining the ascorbic acid content of various crops. This subject has been reviewed recently by Somers and Beeson ('48). Earlier reviews were presented by Giroud ('38), Hamner and Maynard ('42), and Maynard and Beeson ('43–'44). A relationship between illumination and ascorbic acid content has been further substantiated by recent work with strawberries (Ezell et al., '47) and apples (Murneck and Wittwer, '48).

McCollum ('44) studied the relationship between the exposure of tomato fruits to the sun and their ascorbic acid content. He concluded that the amount of illumination received directly by the fruits determined their ascorbic acid content. In the present paper data are presented which support this conclusion. Some implications of this relationship under field conditions are also considered.

EXPERIMENTAL

Experiments with shaded plants in sand culture

Seed of Bonny Best tomatoes were planted in sand on April 7, 1945. They were watered for one week with Hoagland's

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solution no. 1 (Hoagland and Arnon, '38) diluted one to 5. Subsequently they were watered with full-strength Hoagland's solution supplemented with minor elements for the remainder of the experiment. Seedlings were transplanted to two-gallon crocks of sand (one plant per crock) on May 6, and were kept in the greenhouse until June 21. They were watered once a day with Hoagland's solution and flushed once a week with distilled water. On June 21 the plants were moved outside and tied to tall stakes. They were then 10 to 12 in. tall and were good, sturdy, fast-growing plants. Some 98 plants were placed

TABLE 1

The influence of shading for 20 days upon the ascorbic acid content of tomatoes

TREATMENT	8/7/45		8/27/45	
	No. of analyses	Ascorbic acid content [1]	No. of analyses	Ascorbic acid content [1]
Sun	25	23.5 ± 0.50	25	27.6 ± 0.48
Shade	16	17.5 ± 0.55	13	20.2 ± 0.67
Sun to shade	..	.....	25	20.3 ± 0.54
Shade to sun	..	.....	20	27.1 ± 0.60
Sun to leaves in shade	..	.....	25	29.6 ± 0.67
Shade to leaves in shade	..	.....	14	26.7 ± 0.77
Sun to fruits in shade	..	.....	25	24.7 ± 0.53
Shade to fruits in shade	..	.....	11	22.0 ± 1.28

[1] Milligrams per 100 gm fresh weight ± the standard error.

under a muslin-covered shade which reduced the amount of illumination to about 30% of that in full sunlight. These will be called shade-grown plants. The remainder of the plants were grown in full sunlight, and will be called sun-grown plants. All of the plants were pruned to a single stem.

The first ripe fruits were picked from shade-grown and sun-grown plants on August 1. Subsequent pickings followed every few days so that the fruits were analyzed as they ripened.

On August 7 and 8, further variables were introduced into the experiment. Forty-eight plants were transferred from shade to full sunlight and vice versa. In addition, 12 shade-grown plants were moved to full sunlight and all of the leaves and stems enclosed in muslin; only the larger fruits were left

exposed to the sunlight. Another 12 plants were moved to full sunlight, and each of the larger fruit clusters was enclosed in a muslin bag. Either the leaves or the fruits of two groups of sun-grown plants were similarly shaded; that is, one lot was left in full sunlight with only the leaves and stems shaded and the other was left in full sunlight with only the fruits shaded. The remainder of the shade-grown and sun-grown plants were left as controls. The fruits from all plants were harvested and analyzed as they ripened.

Each fruit was analyzed individually. The analytical method used was the indophenol xylene-extraction method described by Nelson and Somers ('45). The results are summarized in table 1 and figure 1. In the figure, each plotted point represents the mean of several fruits. The number of fruits analyzed for two of the harvests is indicated in table 1. For the final harvest each treatment was represented by 20 to 25 fruits. For the other harvests fewer fruits were available for most of the treatments.

In all cases the ascorbic acid content of fruits from shade-grown plants (curve 8, fig. 1) was lower than that of corresponding fruits from sun-grown plants (curve 2) unless the plants were shifted from one environment to another. The ascorbic acid content of fruits from sun-grown plants which had been moved to the shade gradually dropped (curve 7), and those fruits harvested about three weeks after shading had the same ascorbic acid content as those harvested from shade-grown plants. Similarily, shade-grown plants moved into full sunlight (curve 4) produced fruits which gradually increased in ascorbic acid content until, at the end of about three weeks, they equalled those from sun-grown plants in this respect. It is also obvious that shading only the leaves and stems did not lower the ascorbic acid content (curves 1 and 3). In fact, in the case of the sun-grown plants (curve 1), it resulted in a slightly higher ascorbic acid content than that obtained with comparable plants the leaves of which were not shaded (curve 2). However, in the latter case, the leaves probably shaded the fruit to some extent.

Shading only the fruits markedly lowered the ascorbic acid content. Shading only the fruits of shade-grown plants after they were moved into the sun (curve 6) resulted in fruits containing essentially the same amount of ascorbic acid as fruits obtained from shade-grown plants which remained completely in the shade. With sun-grown plants, shading the fruits resulted in a lowering of the ascorbic acid content

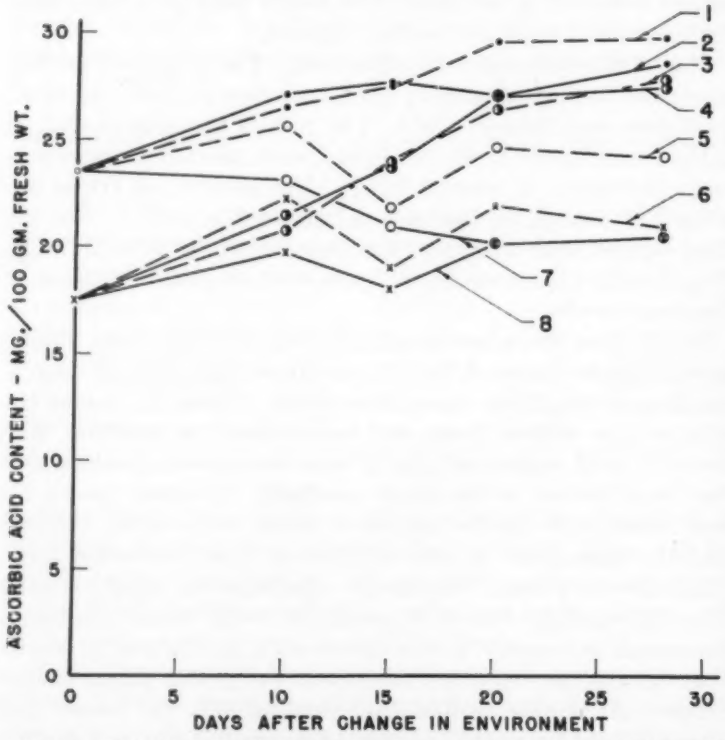

Fig. 1 The influence of shading various parts of the plant upon the ascorbic acid content of tomato fruits. 1: Sun-grown plants with leaves and stems shaded. 2: Sun-grown plants left in the sun. 3: Shade-grown plants with leaves and stems shaded after being moved into the sun. 4: Shade-grown plants moved into full sunlight. 5: Sun-grown plants with only the fruits shaded. 6: Shade-grown plants with only the fruits shaded after being moved into the sun. 7: Sun-grown plants moved into the shade house. 8: Shade-grown plants left in the shade house.

(curve 5), although not to the values obtained for shade-grown plants. There is some reason to believe that the muslin bags may have been less effective in shading the fruits than in the rest of the shading treatments. In this case, the bags were large enough initially to fit loosely over a fruit cluster, but as the fruits developed the cloth was stretched rather taut. This had a tendency to open the weave and may have resulted in a higher transmission of light than that obtained with muslin in the other treatments.

These data seem to show rather clearly that the direct illumination of tomato fruits is essential for the production of a high ascorbic acid content. The relationship between the amount of illumination received by the leaves and the ascorbic acid content of the fruits is less clear-cut. The data obtained by moving shade-grown plants into the sun and shading only the fruits (curve 6, fig. 1) indicate that the illumination of the leaves does not have a significant effect upon the ascorbic acid content of the fruits which ripen subsequently. However, when only the fruits of sun-grown plants were shaded, the ascorbic content of those fruits which ripened subsequently assumed an intermediate value which is significantly higher ($P<0.01$) than that for fruits from sun-grown plants which were completely shaded. One possible explanation for this difference is mentioned above, namely, somewhat less effective shading of the fruits by the muslin in this case. Another possibility may be that the plant as a whole, when grown in the sun, contained relatively larger amounts of ascorbic acid at the time the plants were shifted from one environment to the other, and this was reflected in the higher ascorbic acid content of the fruits which ripened subsequently. Unfortunately, the ascorbic acid content of the foliage was not determined. However, the data of Åberg ('45) show that the ascorbic acid content of tomato leaves is roughly proportional to the amount of illumination they receive, particularly in the case of older leaves. Similar results were obtained by Hamner and Parks ('44) with turnip leaves. Moreover, these data indicate that the ascorbic acid content of leaves changes rather rapidly following a

change in illumination; somewhat more rapidly than was observed in the present experiments with tomato fruits. If this is true, the ascorbic acid content of the leaves of those plants moved from the shade house into full sunlight should have increased rapidly. It seems probable that the ascorbic acid content of the leaves of shade-grown plants moved into the sun approached that of sun-grown plants within a week or so. Still, such an increase was not reflected in the ascorbic acid content of shaded fruits from such plants (curve 6, fig. 1). This leads to the suggestion that probably the higher ascorbic acid content of shaded fruits from sun-grown plants was not due to a higher ascorbic acid content of the leaves of these plants. In other words, it appears that the ascorbic acid content of the fruits is not related to that of the leaves.

Field experiments

A number of varieties of tomatoes were grown in field plots in 8 localities in the United States during the summer of 1945. The seed, in all cases, was from a single source. The plants were grown under the conditions usually practiced at each location. Ripe fruits were shipped to Ithaca for ascorbic acid analyses. At each locality an integrating light recorder was installed which gave a measure of the total amount of visible radiation received on the field. These light recorders were similar to those described by Sprague and Williams ('41), except that the light receiver contained filters which restricted the light striking the phototube (RCA 922) to wave lengths between about 3,700 and 7,500 Angstrom units. All of the recorders did not have the same sensitivity, but before they were sent to the collaborators and again after being returned they were all operated simultaneously with "standard" light records in Ithaca, to obtain comparable calibration data. In this way the individual recorders could be compared with each other. In all cases it was found that the individual recorders had the same relative responses before being sent out and after being returned. As a consequence it is believed that the

records obtained at each locality give a fairly good estimate of the relative amount of visible illumination received.

The relative illumination and ascorbic acid content of the fruits are summarized in tables 2 and 3. Table 2 gives the relative amount of illumination received at each locality for three weeks prior to harvest (Ithaca = 100). Here the "first week" is the one just prior to harvest. (Some of the harvest dates given in the table may be incorrect by a day or so, since harvest dates were not indicated in all cases by the collaborators.)

TABLE 2

Relative illumination for three weeks prior to harvest (Ithaca = 100)

LOCATION	HARVEST DATE	WEEKS PRIOR TO HARVEST			TOTAL FOR TWO WEEKS PRIOR TO HARVEST	TOTAL FOR THREE WEEKS PRIOR TO HARVEST
	1945	*First*	*Second*	*Third*		
Ann Arbor, Michigan	Sept. 10	96	78	177	86	106
Berkeley, Calif.	Sept. 9	110	89	166	99	114
Berkeley, Calif.	Sept. 19–20	76	92	184	84	106
Ithaca, New York	Aug. 22	100	100	100	100	100
Ithaca, New York	Sept. 12	98	82	120	90	96
Knoxville, Tenn.	Aug. 26	70	52	69	61	62
Logan, Utah	Oct. 10	65	69	144	67	84
Pasadena, Calif.	Sept. 6	101	93	149	97	108
Provo, Utah	Sept. 14	105	102	182	103	120
Salinas, Calif.	Sept. 7	114	106	196	109	128
Salinas, Calif.	Oct. 4	30	56	113	43	59

This table also includes the relative total illumination for a two-week and a three-week period prior to harvest. This total is included because previous studies (Hamner et al., '45) and the results cited above indicate that it takes about two to three weeks for a change in illumination to produce significant changes in the ascorbic acid content of the fruit which ripens on tomato vines.

The ascorbic acid content of the fruits is summarized in table 3. The locations listed in the first column of this table are arranged according to the relative total illumination for a three-week period prior to each harvest. The data show that there

TABLE 3

Ascorbic acid content[1] *of tomato varieties grown at various locations*

LOCATION	HARVEST DATE	EARLIANA	MARGLOBE	NORTON	ESSEX WONDER	BONNY BEST	PEARSON	RUTGERS
Salinas	9/7/45	21.4 (23)[1] ± 0.80	21.3 (20) ± 0.93	20.5 (21) ± 1.14	20.8 (19) ± 1.22	17.4 (21) ± 0.70	18.0 (19) ± 0.95	19.4 (13) ± 0.98
Provo	9/14/45	22.3 (18) ± 0.83	20.7 (10) ± 1.35	23.3 (16) ± 1.01	23.0 (20) ± 0.74	23.2 (12) ± 1.08	.....	.....
Berkeley	9/9/45	19.8 (18) ± 0.90	23.1 (18) ± 1.18	23.7 (10) ± 1.33	.....	.....	.....	.....
Berkeley	9/19/45	20.6 (20) ± 0.50	22.7 (18) ± 0.99	23.2 (17) ± 1.20	.....	.....	.....	.....
Ann Arbor	9/10/45	23.8 (21) ± 0.75	25.7 (21) ± 1.01	32.6 (21) ± 1.32	28.6 (21) ± 1.27	.....	.....	.....
Ithaca	8/22/45	23.1 (24) ± 0.55	.....	.....	26.5 (21) ± 0.77	26.1 (21) ± 0.70	.....	.....
Ithaca	9/12/45	24.2 (19) ± 0.87	26.7 (20) ± 0.87	29.9 (20) ± 1.08	30.7 (20) ± 1.12	31.1 (20) ± 0.96	21.6 (20) ± 1.00	24.1 (20) ± 0.77
Logan[2]	10/10/45	23.6 (29) ± 0.70	26.6 (10) ± 1.12	27.7 (14) ± 1.05	.....	.....	17.3 (6) ± 1.33	.....
Knoxville	8/26/45	25.5 (6) ± 1.50	23.7 (14) ± 0.81	27.5 (15) ± 0.88	29.5 (19) ± 0.95	27.5 (17) ± 0.64	18.6 (10) ± 0.91	26.2 (8) ± 1.19
Salinas	10/4/45	.....	24.4 (20) ± 0.78	21.4 (19) ± 1.00	.....	.....	.....	.....

[1] Ascorbic acid cotent is expressed as mean milligrams per 100 gm of fresh fruit ± the standard error, with the number of analyses in parentheses.

[2] Vines partially frozen about three weeks prior to harvest.

apparently was no correlation between the relative illumination three weeks prior to harvest and the ascorbic acid content of the tomato fruits. A study of the data shows that there was also no apparent correlation between illumination one week or two weeks prior to harvest and the ascorbic acid content of the fruits.

DISCUSSION

The above experiments with plants in sand culture indicate that the intensity of illumination is effective in influencing the ascorbic acid content of tomato fruit only insofar as the fruits themselves are directly illuminated. Apparently the intensity of illumination of the vines alone has a smaller effect, if any. Moreover, about three weeks are required to obtain the full effects of a change in illumination. The results in tables 2 and 3 might be explained on the basis of the difference in growing conditions in the various localities. There no doubt existed considerable differences in the degree of foliation and the resultant relative shading of the individual fruits by the leaves. Certainly there appeared to be a considerable difference between the foliation at Ithaca and Provo (as judged by personal observation). At the former location the vines were partially defoliated by foliar diseases, particularly at the second harvest, whereas at the latter location the plants were very large and were densely shaded by leaves. It should be noted that the vines at Logan, Utah, were partially frozen about three weeks prior to the harvest, hence these fruits were probably exposed more during the ripening period than would normally be expected.

Somers, Hamner and Nelson ('45) observed a correlation between the total amount of sunlight about three weeks prior to harvest and the ascorbic acid content of two varieties of tomatoes grown in three fields in upstate New York. The fields were separated by distances up to about 30 miles, and the varieties were John Baer and Stokesdale, which suffered rather extensive defoliation, especially during the latter part of the

season in question. Probably under such conditions, where the vines are at least partially defoliated in all fields, there is a fairly good correlation between the total amount of illumination on the various fields and the direct illumination of fruits produced in those fields. In such a case the ascorbic acid content of the fruits should be correlated with the total amount of sunlight. However, under conditions where there are large differences in the degree of foliation and hence in the shading of the fruits, such as obtained in the experiment presently reported, the ascorbic acid content of the fruits would not be expected to be correlated with the total amount of illumination received at various locations.

SUMMARY

Experiments with tomato plants grown in sand culture show that the amount of illumination of the fruits is of importance in determining their ascorbic acid content. Shaded fruits contain less ascorbic acid than comparable fruits exposed to sunshine. On the other hand, the amount of illumination received by the leaves for a few weeks prior to harvest apparently has much less effect, or possibly no effect at all, upon the ascorbic acid content of the fruits produced.

In view of this relationship, it would be expected that under field conditions where large differences in foliation were obtained at different locations there probably would be little or no correlation between the total amounts of sunlight at these locations and the ascorbic acid content of the tomatoes produced. The variation in the direct illumination of the fruits as a result of the differences in foliation probably would mask any possible effects resulting from variations in the illumination of the foliage. Data obtained from field experiments are presented to support this contention.

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AMINO ACIDS IN THE BLOOD AND URINE OF HUMAN SUBJECTS INGESTING DIFFERENT AMOUNTS OF THE SAME PROTEINS [1]

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The influence of dietary protein on the excretion of amino acids in human urine has not been established with certainty. Milheiro ('27) and Folin and Berglund ('22) determined α-amino nitrogen colorimetrically and found increases in this urinary component as protein-rich food was ingested. Hier ('48) and Eckhardt and Davidson ('49) used microbiological techniques and concluded that diet exerts little influence on the urinary excretion of amino acids by man. That the excretion of single amino acids may vary with diet was suggested by Bonnetti ('45), who determined histidine chemically and reported that the amounts of histidine excreted in human urine paralleled those of the total nitrogen excreted. The present study deals with the effect of different levels of the same proteins on the urinary excretion and blood levels of individual amino acids as determined by microbiological assay procedures.

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EXPERIMENTAL

In a preliminary series of experiments (Steele, '49) three graduate students, two females and one male, were given 25, 50 or 100 gm of protein daily, supplied largely by eggs and cottage cheese. In the main experiment 4 college men served as subjects. The amounts of protein ingested daily were 25, 100 and 200 gm during three successive periods. Approximately 6 gm of the protein were supplied by fruit

TABLE 1

Composition of diets

COMPONENTS	PROTEIN LEVEL		
	25 gm	100 gm	200 gm
	gm	*gm*	*gm*
Beef, ground	50	235	485
Bread	20	94	194
Milk	228	1070	2228
Orange juice	150	150	150
Peaches, canned	150	150	150
Pears, canned	200	200	200
Applesauce	100	100	100
Tomatoes	100	100	100
Potatoes	100	100	100
Lettuce	100	100	100
Butter	60	45	30
Oil	40	40	..
Sugar	60	20	..
Candy [1]	ad lib.	ad lib.	ad lib.

[1] Sugar, butter, H_2O, vanilla.

and vegetables and the remaining protein was contributed by bread, meat, and milk in a ratio of 1:5:4 parts of protein respectively (table 1). The diets were fed to the 4 subjects in the following order: 25 gm protein for 9 days; 100 gm protein for 6 days; 200 gm protein for 6 days.

Sample collections

Twenty-four hour specimens of urine were collected on the last three days of each period and preserved under toluene

in the cold until analyzed for amino acids and total nitrogen. Creatinine was determined on all urine samples as an index of the completeness of collection. Three-day fecal samples were collected in an acetic acid-alcohol mixture with carmine as a marker. The total collection was weighed, homogenized in a Waring Blendor and an aliquot removed for the determination of total nitrogen. Samples of blood were withdrawn by venous puncture, after a 12-hour fast, on the last day of each period.[2] The samples were then placed in centrifuge tubes containing sodium citrate, centrifuged for one-half hour at 3,000 R.P.M., and the plasma removed.

Preparation of samples for analysis

Bread samples were prepared for analysis by drying at 60°C. in a vacuum oven, followed by thorough grinding in a mortar. Two grams of this powder were then hydrolyzed. Meat was dried at 60°C. in a vacuum oven, which was followed by an 8-hour chloroform extraction in a Soxhlet extractor to remove fat. Approximately 0.2 gm were used for hydrolysis. Ten grams of the pooled milk sample and 20 gm of the basal diet [3] were hydrolyzed in preparation for analysis. All food samples were analyzed in duplicate. They were hydrolyzed for 8 hours at 15 lb. pressure in 4 N HCl; for the determination of tyrosine and tryptophan the samples were autoclaved with 4 N NaOH for 15 hours. After autoclaving, the samples were adjusted to pH 6.8, filtered and diluted as necessary.

Urine samples were hydrolyzed by the following procedure: 100- to 250-ml aliquots of the urine were treated with enough concentrated HCl to make the normality roughly 0.1 and the samples were then evaporated *in vacuo* at a temperature that did not exceed 60°C. until they contained approximately 25 mg of α-amino N per 10 ml of concentrate. Five milliliters

[2] We are indebted to Mr. George R. Drysdale for withdrawing all the blood samples used in this experiment.

[3] Orange juice, peaches, pears, applesauce, tomatoes, potatoes and lettuce.

of concentrated HCl were added to 10 ml of this concentrate and the samples hydrolyzed for 4 hours at 15 lb. pressure in the autoclave. The hydrolyzed samples were adjusted to a pH of 6.8 with NaOH, diluted to 50 ml, and filtered through no. 42 Whatman filter paper. For the determination of tryptophan, aliquots of urine were made alkaline with 2 N NaOH and the samples autoclaved for 5 hours at 15 lb. pressure.

Analytical procedures

The hydrolysates of urine or food were analyzed for 18 amino acids by the microbiological methods of Sauberlich and Baumann ('46) and Steele et al. ('49). The assay organisms used were *Leuc. mesenteroides, Leuc. citrovorum* (American Type Culture Collection no. 8081), *Lactob. arabinosus* and *Streptococcus faecalis.* Histidine was also determined chemically by the colorimetric method of Macpherson ('42), and the chromatographic procedure of Bergdoll and Doty ('46) was used to separate histidine from the other substances present in urine. The latter procedure was varied in that for the final washing of the column an excess of the 0.125 M bicarbonate solution was used, removing histidine from the column but leaving arginine. The final treatment with pyridine plus HCl was omitted as both chemical and microbiological determinations for histidine were carried out on the final eluate.

The α-amino nitrogen content of the urine samples was determined by the Cu method of Pope and Stevens ('39) as used by Albanese and Irby ('44) and modified by Clayton et al. ('49). Creatinine was determined by the method of Peters ('43) The total nitrogen of urine, feces, and food samples was determined by the method of Scales and Harrison ('20).

Protein-free filtrates of the plasma were prepared according to the method of Hier and Bergeim ('46), and the microbiological analysis of the filtrate was carried out with the media and the general technique used for the urine and food

hydrolysates except that smaller volumes were used, 0.4 ml per tube instead of the 2.0 ml used ordinarily. Good standard curves and reproducible values for the samples were obtained with the smaller volume, and recoveries of pure amino acids added to plasma before deproteinization were $\pm$ 15%.

RESULTS

Condition of subjects

All of the subjects lost weight during the first few days on the low protein diet although the calories were adjusted individually by varying the sugar, butter and candy in the diet. The weights, however, tended to reach a plateau after the 6th day on 25 gm of protein and they fluctuated only slightly when 100 and 200 gm of protein were ingested daily. For all 4 subjects a negative nitrogen balance during the period of low protein intake was followed by a positive nitrogen balance when the nitrogen intake was increased to 14.3 or 28.4 gm per day.

Amino acids in the urine

The amounts of 18 amino acids ingested and excreted in the urine are given in table 2. Cystine was among the amino acids determined, although it is recognized that the results represent minimum values since there is some loss of cystine upon acid hydrolysis (Yeh et al., '47). Glycine, glutamic acid, histidine, cystine, serine, and aspartic acid, in decreasing order, were the most abundant amino acids in the urine samples, regardless of diet. The excretion of serine appeared to be most directly correlated with dietary intake, varying from an average of 52 mg excreted on the low protein diet to 143 mg excreted when 200 gm of protein were ingested daily, an increase in excretion of 175%. However, the values for serine are maximum since in the presence of the vitamin B_6 group, *Leuc. mesenteroides* P-60, the assay organism used, yields high serine recoveries (Steele et al., '49).

TABLE 2

The dietary intake and urinary excretion of amino acids by human subjects[1] at different levels of protein intake

AMINO ACID	25 GM PROTEIN				100 GM PROTEIN				200 GM PROTEIN			
		Amt. excreted				Amt. excreted				Amt. excreted		
	Ing.[2]	Av.	Range	Excreted	Ing.[2]	Av.	Range	Excreted	Ing.[2]	Av.	Range	Excreted
	gm	*mg*	*mg*	*%*	*gm*	*mg*	*mg*	*%*	*gm*	*mg*	*mg*	*%*
Alanine	1.21	59	28–79	4.9	4.70	92	63–142	2.0	9.44	121	90–150	1.3
Arginine	1.12	18	tr–42	1.6	4.28	28	tr–57	0.7	8.62	18	tr–49	0.2
Aspartic acid	1.64	49	42–57	3.0	5.04	91	51–114	1.8	9.77	97	49–149	1.0
Cystine	0.23	108	81–137	47.0	1.06	123	88–168	11.6	2.19	134	85–182	6.1
Glutamic acid	4.04	287	222–384	7.1	14.57	361	103–470	2.5	29.17	415	200–600	1.4
Glycine	0.94	412	360–462	44.0	3.80	500	309–635	13.1	7.50	433	266–520	5.8
Histidine	0.56	117	84–195	20.8	2.30	192	98–378	8.4	4.60	312	169–560	6.8
Isoleucine	1.93	21	9–30	1.1	8.08	29	13–59	0.4	16.60	32	23–43	0.2
Leucine	1.77	27	8–67	1.5	7.32	39	12–94	0.5	15.04	29	15–61	0.2
Lysine	1.59	36	13–81	2.3	6.48	48	27–84	0.7	13.21	68	49–102	0.5
Methionine	0.47	6	3–11	1.3	2.15	9	4–18	0.4	4.40	8	5–16	0.2
Phenylalanine	0.96	23	8–50	2.4	3.84	26	9–53	0.7	7.83	30	12–48	0.4
Proline	1.97	41	22–66	2.1	7.91	53	28–86	0.7	16.20	58	36–95	0.4
Serine	1.04	52	25–67	5.0	3.91	92	29–189	2.3	7.83	143	56–304	1.8
Threonine	0.84	38	26–56	4.6	3.27	61	34–103	1.9	6.60	68	34–175	1.0
Tryptophan	0.31	31	22–46	10.0	1.16	41	29–70	3.5	2.37	33	22–42	1.4
Tyrosine	1.09	32	16–47	2.9	4.14	46	22–65	1.1	8.33	68	28–95	0.8
Valine	1.02	21	7–53	2.0	4.61	26	8–60	0.6	9.47	27	12–45	0.3

[1] Figures represent 24-hour collection values (average of 4th, 5th, and 6th days) of 4 male subjects fed each level of protein for 6 days. Acid hydrolyzed urine was used for the analyses except in the case of tryptophan, where an alkaline hydrolysis was employed (see text).

[2] Ingested.

Histidine showed the most marked unequivocal variation with diet; the average excretion was 117 mg per 24 hours on a daily intake of 0.56 gm of this amino acid, while 312 mg were excreted daily when the intake was 4.5 gm. This is an increase of 167% in excretion with an approximate 8-fold increase in intake. Alanine excretion increased 102% as the intake of alanine increased from 1.21 gm to 9.44 gm per day (table 2). Tyrosine also was excreted in increasingly higher amounts as the protein intake increased. Nine of the other amino acids — aspartic acid, cystine, glutamic acid, isoleucine, lysine, phenylalanine, proline, threonine, and valine — showed lesser increases in excretion as the intake of protein increased, while the excretion of arginine, glycine, leucine, methionine, and tryptophan actually appeared to be lower on the 200 gm protein diet than on the 100 gm diet.

Of the amino acids determined in the preliminary series (Steele, '49), histidine again showed the greatest increase in excretion with increased protein intake; three subjects excreted an average of 71 mg of histidine per day while ingesting 25 gm of protein and 255 mg daily when 100 gm of the same proteins were ingested.

To ascertain whether the test organism *S. faecalis* was responding only to histidine or to histidine plus other substances, the chemical method of Macpherson ('42) was used to determine this amino acid in two random samples of urine which were also analyzed with *S. faecalis*. The chemical analyses of the urines yielded values of 220 mg and 225 mg of histidine per 24 hours and the microbiological analyses 213 and 213 mg. Certain chromatographic (Bergdoll and Doty, '46) fractions of urine contained 120 and 196 mg of histidine by the chemical method and 144 and 218 mg, respectively, by the microbiological method.

Successive urine samples from subjects H. D. and A. H. on diets containing 100 gm of protein daily and from B. H. on a diet containing 200 gm of protein were assayed individually for 18 amino acids in an attempt to determine the daily variation in amino acid excretion. Glycine, lysine, and

tryptophan tended to be excreted by all three subjects at a fairly constant level for the three days (table 3), while certain other amino acids such as alanine, cystine, and threonine showed relatively large day-to-day variations. The greatest fluctuations were encountered in specimens from subject H.

TABLE 3

The daily excretion of amino acids by three male subjects ingesting a controlled diet (all values in mg/24 hours)

AMINO ACID	SUBJECT H. D.[1]			SUBJECT A. H.[1]			SUBJECT B. H.[2]		
	Day [3]			Day [3]			Day [3]		
	1	2	3	1	2	3	1	2	3
Alanine	81	85	95	83	125	142	160	132	142
Arginine	39	43	57	tr	tr	tr	tr	tr	tr
Aspartic acid	70	102	110	89	114	111	86	87	75
Cystine	88	154	168	123	106	104	145	85	123
Glutamic acid	..	436	410	343	470	440	430	332	420
Glycine	309	580	485	635	565	520	500	480	520
Histidine	130	192	183	322	362	378	161	185	160
Isoleucine	59	37	46	18	21	17	23	28	25
Leucine	64	63	94	20	21	22	27	15	27
Lysine	81	84	90	42	44	47	56	56	58
Methionine	13	18	16	6	8	4	9	5	7
Phenylalanine	49	43	66	27	24	26	24	28	25
Proline	62	72	86	47	43	48	57	50	58
Serine	88	70	106	199	163	206	97	69	87
Threonine	51	72	79	78	84	103	38	29	52
Tryptophan	30	31	35	64	70	75	34	35	35
Tyrosine	55	63	70	68	62	65	49	57	54
Valine	41	50	60	14	8	12	21	26	21

[1] One hundred grams of protein daily.

[2] Two hundred grams of protein daily.

[3] Collections were made on the last three days of the 6-day protein period.

D., who excreted more of these amino acids on the third collection day than on the first, without any change in the excretion of creatinine or total N nor any parallel change in the total excretion of total α-amino N as determined by the Cu method.

In agreement with others (Steele et al., '47; Dunn et al., '47; Woodson et al., '48), individual subjects on comparable

diets were found to differ from one another in their excretion of the amino acids. Subject H. D. usually excreted the greatest amount of any amino acid in comparison with the other three subjects, and his excretion of isoleucine, leucine, lysine, methionine, phenylalanine, and valine was particularly high. Often the amounts of these amino acids excreted by H. D. were 4 times as great as the highest amount excreted by any of the other three subjects. Thompson and Kirby ('49) have reported a positive correlation between the body weight of human subjects and the excretion of threonine, leucine and arginine, though not of lysine. Subject H. D., however, with the highest excretion values for most of the amino acids, weighed 15 kg less than D. B., the heaviest subject, who excreted less of most of the amino acids than the other subjects.

Amino acids in blood

Of the amino acids determined, alanine, glutamic acid, lysine, and valine were present in the plasma in the highest amounts on all three levels of protein fed, 31 to 50 μg per milliliter of plasma (table 4); glycine, methionine, and aspartic acid were present in the lowest amounts, 8 to 16 μg per milliliter; and the other amino acids were present in intermediate amounts. Cystine and serine were not determined with sufficient accuracy for classification.

Increases in the amounts of protein ingested usually had little effect on the fasting level of the amino acids in the plasma, although leucine varied in one subject from 19 μg per milliliter on the low protein diet to 27 μg per milliliter of plasma on the highest level, and in a second subject from 17 μg to 29 μg per milliliter. Proline, tryptophan, lysine and valine showed occasional increases in the plasma with the increased protein intake. There were some variations in the amino acid content of the plasmas from different individuals, but in general subjects having low values on one diet continued with low values for the entire experiment.

DISCUSSION

In agreement with previous workers, the present authors found that diet had relatively little influence on the amounts of most amino acids in human urine. In the present experiment, however, the urinary excretion of serine, histidine, alanine, and tyrosine increased significantly as the intake of

TABLE 4

Fasting concentrations of amino acids in the plasma of human subjects fed different levels of protein (all values expressed as μg/ml)

AMINO ACID	25 GM PROTEIN		100 GM PROTEIN		200 GM PROTEIN	
	Individual	Av.	Individual	Av.	Individual	Av.
Alanine	40–53–56–49	50	48–34–49–35	42	33–36–46–32	37
Arginine	28–(—)–28–(—)	28	(—)–36–33–16	28	37–29–29–21	29
Aspartic acid	12–(—)–10–9	10	(—)–9–6–9	8	10–14–8–8	10
Cystine [1]						
Glutamic acid	41–44–45–45	44	42–40–47–45	44	32–48–47–35	41
Glycine	14–11–14–16	14	9–9–8–16	11	14–6–8–10	10
Histidine	28–23–23–27	25	21–20–22–20	21	17–24–29–20	23
Isoleucine	21–19–20–20	20	21–16–17–18	18	22–23–17–15	19
Leucine	29–19–17–20	21	26–24–20–25	24	33–27–29–23	28
Lysine	37–28–37–45	37	34–37–42–34	37	34–37–53–49	43
Methionine	8–10–9–11	10	10–8–11–8	9	9–9–9–8	9
Phenylalanine	20–20–15–22	19	23–16–19–16	19	20–20–24–15	20
Proline	27–(—)–24–26	26	(—)–30–26–27	28	31–38–29–31	32
Serine [2]						
Threonine	35–32–29–33	32	39–30–28–27	31	25–26–28–20	25
Tryptophan	16–14–10–18	15	17–16–17–18	17	22–16–23–18	20
Tyrosine	12–(—)–15–13	13	(—)–14–15–9	13	14–5–15–10	11
Valine	35–(—)–26–32	31	37–38–28–34	34	40–27–49–41	39

[1] Values range from zero to trace.

[2] Vitamin B_6 interferes.

protein was increased. Histidine excretion showed a relatively high increase, 167%, as the dietary protein was increased 8-fold. In a study by Hier ('48) the excretion of most of 13 amino acids was essentially the same on a non-protein diet as on one supplying 14.7 gm of protein per day; however, the excretion of aspartic acid, glutamic acid, histidine, methionine, threonine, tryptophan, tyrosine, and valine

increased somewhat as the protein intake was increased from 11.1 to 14.7 gm. Histidine excretion increased the most, 45%.

In the present experiment there were wide variations among the amounts of the 18 amino acids excreted daily. The only amino acid that was abundant both in the diet and in the urine was glutamic acid; for the other amino acids no correlation between intake and excretion was evident. It may be significant that glycine, glutamic acid, histidine, cystine and aspartic acid, the amino acids occurring in large amounts in the urine, are "non-essential" for the adult organism, whereas the so-called "essential" amino acids were present in lower amounts. Of the 5 non-essential amino acids referred to, 4 are classed as glycogenic, while the status of histidine is uncertain. Thus the high excretion of these amino acids may be related to their glycogenic properties, or to the fact that many of them can be formed within the body from carbohydrate fragments.

There was little correlation between the concentrations of the amino acids in fasting plasma and the amounts excreted daily. Glycine was excreted in large amounts in the urine but occurred in only small quantities, approximately 13 μg per milliliter, in the plasma. Threonine was excreted in small amounts in the urine, 38 to 68 mg per 24 hours, but occurred in the plasma in relatively high concentration, higher than that of histidine which was fairly abundant in urine. In agreement with Brewer et al. ('47), Schmidt ('29), and Friedberg and Greenberg ('47), it appears to be difficult to alter the "fasting" amino acid levels in human blood by diet. Man apparently responds differently to diet than the rat, since Wiss ('48, '49) and Wiss and Krueger ('48) using plasma collected from fasted rats, have reported definite variations in levels of amino acids, caused by diet.

Fasting values, however, give no indication of any relationship that may exist between amino acids in the blood and those appearing in the urine when the subject is in a postprandial state. Harper and Uyeyama ('48) have shown that subjects given DL-methionine orally exhibit a definite peak

in blood amino acid levels of methionine in from one to two hours after its administration, after which normal levels are gradually approached in the manner of glucose tolerance curves. Gutman and Alexander ('47) administered 1 gm of glycine per 10 lb. of body weight to human subjects and found that only a small part of this ingested amino acid, 38 and 43 mg, was recovered in the urine in three and one-half and 5 hours, respectively. Thus, in the case of glycine, urine levels would seem not to be greatly influenced by a post-prandial rise in plasma glycine.

It would appear from the present data and those of others (Dunn et al., '47; Eckhardt and Davidson, '49; Hier, '48) that the human subject does not treat all amino acids equally in such metabolic processes as deamination and transamination. As was pointed out by Sheffner et al. ('48), the renal tubules must possess a marked selectivity in their normal reabsorptive function. These workers found that the average renal clearance of histidine for two subjects was 9.6 mg of plasma cleared per minute, while the average renal clearances of threonine, lysine, and methionine were between 1 and 2 ml and for leucine, isoleucine, and valine were less than 1 ml of plasma cleared per minute.

SUMMARY

1. Four college students were placed on diets supplying 25, 100 and 200 gm of protein daily contributed by meat, milk, and bread, and the urinary excretion and blood levels of amino acids were determined microbiologically.

2. The excretion of histidine, serine, alanine, and tyrosine increased rather markedly with an increase in dietary protein, but not proportionally with the intake. Increasing the intake of protein had little effect on the excretion of most other amino acids or on the "fasting" levels of the individual amino acids in the plasma.

3. The non-essential amino acids histidine, glutamic acid, cystine, aspartic acid, alanine and serine occurred most abundantly in acid-hydrolyzed urine. The essential amino

acids were excreted in much smaller amounts. The amounts of the amino acids found in the plasma during the fasting state did not parallel the amounts excreted in the urine over a 24-hour period.

4. Much individual variation in the excretion of amino acids was evident. This did not seem to be correlated with body weight, or with the excretion of creatinine, α-amino nitrogen or total nitrogen.

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EFFECT OF XANTHOPHYLL ON THE UTILIZATION OF CAROTENE AND VITAMIN A BY THE RAT[1]

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The utilization of carotene and vitamin A by man and by a variety of animals is affected by many factors, as is shown through the work of numerous investigators. However, little attention has been given to the possible effects of non-provitamin A xanthophylls on the utilization of carotene and vitamin A, although several of these substances have been extensively described with regard to their occurrence and significance in plants (Strain, '38). This is of interest because the xanthophylls are closely related structurally to the carotenes and they always occur with such provitamins A in plants, and in greater abundance.

On the basis of known antagonistic relationships between a number of nutritional factors and their structural analogs (Woolley, '47), it seems possible that xanthophylls may antagonize the utilization of provitamin A carotenes. Kemmerer et al. ('47) found that a mixture of xanthophylls, in a concentrate from spinach, decreased the storage of vitamin A in the liver of vitamin A-deficient rats supplemented with

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carotene, which indicates that such an antagonistic relationship may exist. These findings were confirmed and extended by Kelley and Day ('48a) who, in a preliminary report, stated that relatively large amounts of xanthophyll (lutein) impair the utilization of vitamin A itself, as well as carotene. Johnson and Baumann ('48) also found that large amounts of lutein decrease the utilization of carotene. However, using weight gain and mortality as criteria, Sherman ('47) observed that small amounts of lutein seemed to have a carotene-sparing action when given to rats on a vitamin E-low diet.

This paper presents the supporting evidence for the statements in the earlier preliminary report (Kelley and Day, '48a), and gives additional observations on this subject.

EXPERIMENTAL

The crystalline carotene [3] was 90% β- and 10% α-carotene. The vitamin A concentrate [4] contained 200,000 U.S.P. units per gram. Xanthophylls from two different sources were employed. A crystalline preparation [5] was recrystallized by dissolving it in chloroform and flooding the chloroform solution with 5 volumes of purified Skelly-solve B. The crystals which formed were isolated and dried in a vacuum desiccator over phosphorus pentoxide and paraffin. The absorption curve of a chloroform solution of this material, determined on a Beckmann spectrophotometer, showed sharp maxima at 445 and 480 mμ. This preparation was principally lutein because purified lutein in chloroform has maxima at 456 and 487.5 mμ (Strain, '38). Another source of xanthophyll was a concentrate [6] from which the crystalline product was isolated by the following procedure: the fatty concentrate was saponified with 30% potassium hydroxide in methanol; the saponified material was extracted with ether; and the ether extract was washed, dried, and evaporated to a small volume. This con-

[3] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.
[4] Kindly donated by Distillation Products, Inc., Rochester, N. Y.
[5] Obtained from the American Chlorophyll Co., Alexandria, Va.
[6] Obtained from the Midwest Extraction Co., Rockford, Ill.

centrated solution was flooded with 5 volumes of purified Skelly-solve B. The crude separated crystals were further purified by being dissolved in a minimum quantity of purified dioxane, and precipitated by flooding with 5 volumes of purified Skelly-solve B. The resulting crystals were isolated and dried over phosphorus pentoxide and paraffin as before. The absorption spectrum of an ethanolic solution of these crystals showed sharp maxima at 420 and 444 mμ. Further recrystallization by this procedure produced no change in the absorption characteristics. Purified lutein has absorption maxima at 420 and 446.5 mμ when dissolved in ethanol (Strain, '38). Although the evidence indicates that both preparations were largely lutein, they will be referred to as xanthophyll.

Suitable quantities of the carotene, vitamin A concentrate, and xanthophyll were each dispersed in fresh cottonseed oil [7] periodically and each was kept at 4°C. in low actinic containers.

Weanling albino rats were depleted of their vitamin A stores on a vitamin A-free diet of the following percentage composition: vitamin A-free casein 18, glucose 58, salts 4 (Jones and Foster, '42), dried brewers' yeast 8, irradiated yeast [8] 2, and hydrogenated cottonseed oil [9] 10. When the growth curve began to show a plateau and there was some xerophthalmia the rats were grouped on the basis of sex and weight and placed in individual cages.

The supplements were administered daily by pipetting the material onto a small amount of the basal diet in a glazed earthen cup. In all cases each animal received a total of 0.5 ml of oil per day. The food was removed from the cages approximately 5 hours before feeding the supplements and when all the supplements had been consumed the animals were allowed the food ad libitum. The kind of supplement administered had no influence on the rate at which food was consumed. As a rule the supplements were consumed within 30 minutes.

[7] Wesson oil.
[8] Fleischmann's irradiated yeast no. 360.
[9] Crisco.

The livers and, in some cases, the kidneys were analyzed for vitamin A by the method of Sobel and Werbin ('46); the procedure was the same as that used previously by Kelley and Day ('48b).

Effect of xanthophyll on the utilization of carotene for deposition of vitamin A in the liver

Vitamin A-depleted rats which had been given a daily supplement of 20 μg of β-carotene with 400 μg of xanthophyll for 27 to 32 days stored less vitamin A in their livers than did those given exactly the same amount of carotene alone. This is shown in table 1, series 1 and 2. When the period of supplementation was only 14 days and the carotene was decreased to 15 μg per day, much less vitamin A was deposited in the liver, as is shown in table 3. Also, at this lower level of carotene, 400 μg of xanthophyll in addition, given each rat daily, resulted in the deposition of even less vitamin A than in the case of rats given the carotene without xanthophyll.

The xanthophyll had no significant effect on the rate of growth response to carotene when the latter was administered either at the 15 μg level or the 20 μg level.

Effect of xanthophyll on the utilization of vitamin A for deposition in the liver and kidneys

Rats on the vitamin A-free diet were given daily supplements of 14 μg of vitamin A and 400 μg of xanthophyll, and compared with similar rats given 14 μg of vitamin A alone. The results are shown in series 3 to 8, table 1. In all cases those animals which had received the xanthophyll in addition to vitamin A stored less vitamin A than did those receiving the vitamin A alone. This difference occurred in the kidneys as well as in the liver. In series 7 and 8 the values for vitamin A are considerably lower than those in the other series. The vitamin A supplement used in these two series was prepared several weeks prior to its use, and apparently it had decreased

TABLE 1

Effect of xanthophyll (X) on the content of vitamin A in the liver and kidneys of vitamin A-depleted rats following supplementation with β-carotene (C) or with a vitamin A concentrate (A)

SERIES	DAILY SUPPLEMENT	DAYS ON SUPPLEMENT	NO. AND SEX OF RATS	BODY WT. WHEN SACRIFICED	TOTAL VITAMIN A	
					Liver	Kidney
				gm	μg	μg
1	20 μg C	27	6 (0M)	142 ± 16.6 [1]	25.0 ± 2.8 [1]	.....
	same plus 400 μg X	27	6 (0M)	134 ± 8.8	10.7 ± 1.7	.....
2	20 μg C	32	4 (0M)	168 ± 23.7	32.0 ± 9.2	.....
	same plus 400 μg X	32	5 (0M)	135 ± 13.3	2.6 ± 1.8	.....
3	14 μg A	28	3 (0M)	149 ± 15.3	58.6 ± 24.2	51.0 ± 30.5 [1]
	same plus 400 μg X	28	3 (0M)	139 ± 31.1	37.7 ± 4.1	10.0 ± 3.1
4	14 μg A	32	3 (2M)	179 ± 24.8	31.0 ± 2.8	30.0 ± 5.8
	same plus 400 μg X	32	3 (1M)	173 ± 22.8	22.3 ± 5.5	11.3 ± 7.2
5	14 μg A	32	3 (1M)	179 ± 22.8	38.0 ± 14.1	8.0 ± 1.2
	same plus 400 μg X	32	5 (2M)	139 ± 20.4	26.4 ± 5.6	3.7 ± 1.4
6	14 μg A	39	4 (0M)	172 ± 7.9	32.3 ± 3.4	6.0 ± 0.8
	same plus 400 μg X	39	5 (0M)	172 ± 6.8	23.2 ± 1.2	2.2 ± 0.7
7 [2]	14 μg A	40	5 (3M)	181 ± 20.4	16.5 ± 4.5	8.0 ± 1.8
	same plus 400 μg X	40	5 (3M)	173 ± 10.8	8.0 ± 4.8	2.8 ± 1.4
8 [2]	14 μg A	41	4 (4M)	230 ± 20.0	10.5 ± 2.2	11.8 ± 4.3
	same plus 400 μg X	41	4 (4M)	234 ± 14.5	6.2 ± 2.4	8.5 ± 2.7

[1] Standard error of the mean.

[2] The rats in series 7 and 8 received somewhat less than 14 μg vitamin A per day owing to partial inactivation of the preparation. This was not recognized until it was too late to determine the potency.

considerably in potency. Unfortunately an analysis of the sample was not made immediately before beginning its use.

The average body weights of the animals at the end of the experimental period are included in table 1. The average weights of the animals in the two groups of each series were essentially the same at the beginning of the supplementation period. Thus any differences in the final weights might be attributed to the presence of the xanthophyll. Although in nearly all the series the average gain was less for rats given xanthophyll with the vitamin A, the differences were not significant. Therefore it may be concluded that under these conditions the impairment of vitamin A utilization for storage was not great enough to retard the growth response of the animals to the vitamin A supplements.

Effect of xanthophyll on the rate of depletion of vitamin A stores

Because xanthophyll administered with carotene or vitamin A caused a decrease in the amount of vitamin A stored in the tissues, it seemed of value to determine whether the effect was exerted on the vitamin A in the tissues or previous to its deposition there. If the effect is exerted before the vitamin reaches the tissues, xanthophyll given by mouth in massive doses should not affect the rate of disappearance of vitamin A already present in the tissue stores.

Young adult stock rats were restricted to the vitamin A-free diet and given 2,500 U.S.P. units of vitamin A divided into two equal doses. The animals were then divided into two groups, one of which was given a daily supplement of 400 μg of xanthophyll in 0.5 ml of cottonseed oil and the other an equal amount of cottonseed oil alone. The supplements were administered for 14 days. As is shown in table 2, the concentration of vitamin A was found to be approximately the same in the liver and kidneys of those animals receiving the xanthophyll as in those of the controls given no xanthophyll. These results indicate that the effect of the xanthophyll

TABLE 2

Effect of xanthophyll (X) on the rate of depletion of liver and kidney vitamin A in stock rats. (Each rat was given approximately 2,500 U.S.P. units of vitamin A concentrate in two equal doses over a period of 24 hours before beginning the supplements)

SERIES	DAILY SUPPLEMENT FOR 14 DAYS	NO. AND SEX OF RATS	BODY WT. WHEN SACRIFICED	TOTAL VITAMIN A	
				Liver	Kidney
			gm.	μg	μg
1	0.5 ml cottonseed oil	3 (2M)	234 ± 38.2 [1]	380 ± 69.1	26.3 ± 4.4
	same plus 400 μg X	4 (3M)	273 ± 32.4	407 ± 44.7	22.0 ± 1.2
2	0.5 ml cottonseed oil	3 (0M)	209 ± 11.7	557 ± 64.9	15.0 ± 6.3
	same plus 400 μg X	4 (0M)	200 ± 13.1	525 ± 60.5	11.5 ± 3.6
3	0.5 ml cottonseed oil	6 (3M)	139 ± 9.1	241 ± 23.4	7.2 ± 2.9
	same plus 400 μg X	6 (3M)	144 ± 12.5	264 ± 22.1	9.8 ± 1.4
4 [2]	0.5 ml cottonseed oil	5 (4M)	117 ± 2.9	116 ± 6.8	.....
	same plus 400 μg X	5 (3M)	131 ± 3.9	118 ± 4.9	1.6 ± 1.3

[1] Standard error of the mean.

[2] The rats in series 4 received somewhat less than 2,500 U.S.P. units per animal because the preparation of vitamin A for this series became partially inactivated before it was used. This was not discovered until too late to determine the change in the vitamin A potency.

is exerted previous to the deposition of vitamin A in the tissues, possibly in the alimentary tract.

Effect of vitamin E on the inhibition of carotene utilization by xanthophyll

Because the action of xanthophyll appeared to occur in the alimentary tract it was thought that the pigment might render the intestinal anti-oxidants unavailable for the protection of carotene and vitamin A from destruction, especially if the concentration of such protectors was limited. Therefore, observations were made of the effect of generous supplements of α-tocopherol in sparing carotene fed with xanthophyll.

Vitamin A-deficient rats were divided into three groups and their diets supplemented with (a) carotene, (b) carotene and xanthophyll, or (c) carotene, xanthophyll and α-tocopheryl acetate. Two different series were run. The results

are given in table 3. The concentration of vitamin A in the liver was slightly higher for the group receiving the vitamin E as compared with those receiving carotene and xanthophyll but no vitamin E. However, this difference is no greater than would be expected to result from the addition of α-tocopherol to the carotene supplements (Hickman et al., '44). This evidence therefore indicates that the inhibiting action of xanthophyll is unrelated to the amount of vitamin E ingested.

TABLE 3

Comparison of the effect of vitamin E and of xanthophyll (X) on the content of vitamin A in the liver of vitamin A-depleted rats supplemented with β-carotene (C)

SERIES	DAILY SUPPLEMENT FOR 14 DAYS	NO. AND SEX OF RATS	BODY WT. WHEN SACRIFICED	LIVER VITAMIN A	
				Total	
			gm		*μg/gm*
1	a. 15 μg C	2 (1M)	243 ± 35.4 [1]	10.5 ± 3.5	0.93 ± 0.3
	b. same plus 400 μg X	3 (2M)	217 ± 39.3	1.3 ± 1.1	0.10 ± 0.1
	c. same as b. plus 5 mg α-tocopheryl acetate [2]	3 (2M)	227 ± 35.6	2.7 ± 2.9	0.34 ± 0.4
2	a. same as 1 a.	6 (5M)	208 ± 15.7	9.3 ± 1.8	0.90 ± 0.2
	b. same as 1 b.	5 (4M)	210 ± 18.5	4.9 ± 2.3	0.56 ± 0.4
	c. same as 1c.	4 (4M)	228 ± 7.1	7.4 ± 3.0	0.60 ± 0.3

[1] Standard error of the mean.

[2] Furnished through the courtesy of Merck and Company, Rahway, New Jersey.

DISCUSSION

The evidence presented here shows that large amounts of xanthophyll do not hasten the depletion of liver and kidney vitamin A. The 14-day period for administration of the xanthophyll must be regarded as long enough because Goerner ('38) demonstrated a marked depletion of liver vitamin A when dibenzanthracene was given for only one week. Therefore it may be supposed that the adverse effect on carotene and vitamin A utilization occurs in the alimentary tract. This view is supported by the fact that little if any of the xanthophyll used is absorbed as such by the rat. In these experiments

the blood and tissues of rats fed the xanthophyll never contained more than traces of the pigment.

Adequate data are not available on which to judge whether or not the xanthophyll effect is due to increased excretion of carotene and vitamin A. Johnson and Baumann ('48) attempted to determine the effect of lutein on the excretion of β-carotene but the experimental problem was difficult and the results were equivocal.

Owing to the structural similarities between lutein and β-carotene it was thought by the authors at the beginning of this investigation that lutein might compete with carotene as a substrate in the enzymatic conversion of the latter to vitamin A, thus impairing the formation of vitamin A. Apparently this view was cautiously entertained by Kemmerer et al. ('47). The hypothesis was compatible with the evidence that such conversion occurs in the intestines (Wiese et al., '47; Glover et al., '47) and that the deleterious effect of xanthophyll seems to be confined to the alimentary tract, as is indicated here. However, the marked impairment in the utilization of preformed vitamin A (table 1) is inconsistent with this hypothesis. Therefore it must be concluded that xanthophyll has no specific effect on the enzymatic conversion of carotene to vitamin A.

The evidence available up to this time offers no satisfactory explanation for the seemingly contradictory findings of Sherman ('47). However, in the investigations reported here the diet contained vitamin E and the xanthophyll: carotene ratios were much larger than in Sherman's experiments. The investigations are being continued.

SUMMARY

Large amounts of xanthophyll (lutein) administered with β-carotene or vitamin A decrease the utilization of these substances for tissue storage of vitamin A in rats. They have no effect on the rate of disappearance of vitamin A already present in the tissues, indicating that the inhibitory

action may occur in the alimentary tract. The inhibitory effect is not overcome by extra vitamin E administered as α-tocopheryl acetate.

The effect of the xanthophyll is not due to a specific impairment of the enzymatic mechanism for the conversion of carotene to vitamin A.

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VITAMIN B_{12} AND AMINO ACIDS AS SUPPLEMENTS TO SOYBEAN OIL MEAL AND COTTONSEED MEAL FOR GROWING CHICKS

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Plant proteins from different sources have been used extensively in poultry diets for a long time when supplemented with proteins from animal sources. Many investigations have been carried out on soybean oil meal as a protein supplement for growing chicks and the value of large amounts of it in practical poultry diets is universally recognized. With the discovery and isolation of vitamin B_{12} it is now possible to obtain an optimum rate of growth in chicks with soybean oil meal as the only source of protein in the diet.

Relatively few studies have been carried out with cottonseed meal as the sole source of protein for growing chicks. It has been used successfully when supplemented with other protein concentrates such as fish meal, sardine meal, dried skim milk and meat scraps (Hartwell and Lichtenthaeler, '14; Berry, '34; Barnett and Polk, '42; Ringrose and Morgan, '38; Sherwood and Couch, '39, '40; and Ackerson et al., '38). Draper and Evans ('44) reported that soybean oil meal had a higher nutritive value than any combination of soybean oil meal and cottonseed meal. The results with combinations of herring fish meal and cottonseed meal were similar to those with soybean and cottonseed meals. Grau ('46) investigated cottonseed meal as the sole source of protein for growing chicks in a synthetic type of diet by the method described by

Grau and Almquist ('43). A commercial type of diet gave a growth rate of 7.0% by this method. The addition of lysine alone to cottonseed meal increased the growth rate from 4.4% to 5.7%, and the addition of both lysine and methionine resulted in a rate of 6.9%. Methionine alone had no effect.

Large amounts of commercially prepared cottonseed meal in poultry rations are not recommended because of the toxic effect of gossypol or some other substance which is present in the pigment glands (Eagle et al., '48).

In preliminary studies on the value of cottonseed meal for growing chicks it was found that diets which contained 25 to 40% of cottonseed meal and 5% of sardine meal supported a normal rate of growth. Meat scraps, dried skim milk and fish solubles were less effective than sardine meal in supplementing the cottonseed meal. While these experiments were in progress vitamin B_{12} became available and it was possible to investigate the value of protein concentrates with a more highly purified diet. The results of such studies with soybean oil meal and with cottonseed meal are described in this report.

EXPERIMENTAL

Crossbred chicks (White Leghorn females, New Hampshire Red males) were used throughout the investigation. The dams were kept in batteries so that they did not have access to feces or litter and they received a diet low in vitamin B_{12} for a sufficient period of time to reduce the store of this vitamin in the chicks to a low level. Day-old chicks were placed in electrically heated batteries and were given the various diets and water ad libitum. The experimental diets contained 20% of protein. The protein content ($N \times 6.25$) of each ingredient which contributed to the total protein was determined before it was mixed in the diet. When a new lot of milo or protein concentrate was used, the milo and protein concentrate or the cerelose and protein concentrate were adjusted so that the diet contained a total of 20% protein. This was also true when sardine meal was added to the diet. The composition

of a diet typical of that which contained milo, diet 1, and of that which contained cerelose, diet 2, is given in table 1. The observations on the chicks which received the various diets and supplements are summarized in table 2. When amino acids were tested as supplements to cottonseed meal there were three to 5 chicks per group in each series. The other tests were carried out with 8 to 12 chicks per group in each series. Every test, with the exception of that involving the group which received a mixture of soybean and cottonseed meals and no vitamin B_{12}, group 14, was repeated at least once.

TABLE 1

Composition of typical diets

CONSTITUENTS	TYPICAL DIET		VITAMINS ADDED PER 100 GM	TYPICAL DIET	
	1	2		1	2
	gm	*gm*		*mg*	*mg*
Ground milo	61.5	..	Alpha-tocoperhol acetate	..	2.5
Cerelose	...	48	Menadione	2.5	2.5
Soybean or cottonseed meal	35	47	Thiamine hydrochloride	0.4	0.4
			Riboflavin	0.4	0.4
Mineral mixture [1]	...	3	Pyridoxine hydrochloride	0.4	0.4
Iodized salt	0.5	..	Calcium pantothenate	2.0	2.0
Calcium carbonate	...	1	Niacin	5.0	5.0
Steamed bone meal	2.0	..	Choline chloride	200.0	200.0
Fish liver oil	1.0	1.0	Folic acid	0.2	0.2
$MnSO_4H_2O$	0.04		Biotin	..	0.02

[1] Richardson and Hogan, '46.

The gossypol content of the various batches of cottonseed meals ranged from 0.033 to 0.04%. These meals were not toxic at a level of 50%. One batch which contained 0.116% of gossypol was very toxic at a level of 35%. A charcoal adsorbate [1] was used as a source of vitamin B_{12}; it contained the equivalent of 2 mg of vitamin B_{12} per pound.

Vitamin B_{12} as a supplement to soybean oil meal

The average weights of chicks which received 0.0, 1.0 and 5.0 gm of charcoal adsorbate of vitamin B_{12} per kilogram of

[1] Prepared by Merck and Company and found by their assay to contain the equivalent of 2 mg of vitamin B_{12} per pound.

TABLE 2

Vitamin B_{12} as a supplement to soybean oil meal (A); vitamin B_{12} and amino acids as supplements to cottonseed meal (B); and soybean oil meal as a supplement to cottonseed meal for growings chicks (C)

GROUP NO.	SUPPLEMENT	CHARCOAL ADSORBATE	CHICKS OBSERVED			AVERAGE WEIGHT — WEEKS						"t" value [1]
			Total	Males	Females	Males			Females			
						2	4	6	2	4	6	
		gm/kg	no.	no.	no.							
	(A) VITAMIN B_{12} AS SUPPLEMENT TO SOYBEAN OIL MEAL											
	Basal = Milo-soybean oil meal											
1	Basal		51	19	32	99	222	382	90	171	263	
2	Vitamin B_{12}	1	45	20	25	101	249	488	94	233	435	8.1**
3	Vitamin B_{12}	5	30	13	17	110	268	496	106	251	435	8.47**
	Basal = Cerelose-soybean oil meal											
4	Basal		35	16	19	119	273	453	101	217	338	
5	Vitamin B_{12}	1	26	18	8	116	264	451	113	264	433	1.71
6	Vitamin B_{12}	5	29	11	18	118	306	530	112	262	435	2.45*
7	Sardine meal 5%		27	10	17	121	274	493	118	257	455	2.98**
	(B) AMINO ACIDS AND VITAMIN B_{12} AS SUPPLEMENTS TO COTTONSEED MEAL											
	Basal = Cerelose-cottonseed meal											
8	Basal	5	25	10	15	94	208		92	183		
9	DL-Lysine 0.8%		14	2	12	115	256		109	226		3.66**
10	DL-Lysine 0.8%	5	12	5	7	123	277		130	304		8.37**
11	All combinations of lysine	5	48	24	24	126	296		124	286		12.08**
12	DL-Lysine 0.8% DL-Methionine 0.2% DL-Tryptophan 0.1%	5	20	10	10	128	302		121	270		9.73**
13	Sardine meal 5%		12	5	7	132	320		111	273		7.11**
	(C) SOYBEAN OIL MEAL AS SUPPLEMENT TO COTTONSEED MEAL											
	Basal = Cerelose-soybean oil meal 20%, cottonseed meal 30%											
14	Basal		8	3	5	91	236	406	84	227	323	
15	Vitamin B_{12}	5	19	5	14	124	289	508	116	264	427	2.59*

[1] The "t" values refer to the final weights in each series and are for comparison with the basal diet.
* = Significant to 0.05 and ** = significant to 0.01.

diet as a supplement to a milo-soybean oil meal and a cerelose-soybean oil meal diet are given in section A of table 2. There it can be seen that 1 gm of the concentrate or an amount equivalent to about 5 μg of crystalline vitamin B_{12} as a supplement to the milo diet supported as rapid a rate of growth as 5 gm. When the diet contained cerelose, 5 gm gave a slightly faster rate of growth than 1 gm. The addition of 10 gm of adsorbate to either diet did not increase the rate of growth over that obtained with 5 gm. These data show that the amount of vitamin B_{12} supplied by 1 to 5 gm of charcoal adsorbate was sufficient for a normal rate of growth in chicks when the diet contained milo with soybean oil meal as the only protein concentrate. The smaller amount may be slightly inadequate with the cerelose diet. The vitamin B_{12} was as effective in supplementing the soybean oil mean as 5% of sardine meal.

Amino acids and vitamin B_{12} as supplements to cottonseed meal

The data showing the effect of supplementing cottonseed meal with vitamin B_{12} and amino acids are given in section B, table 2. The chicks (group 10) which received vitamin B_{12} and 0.8% of DL-lysine grew as rapidly as those which received the same basal diet (group 13) supplemented with 5.0% of sardine meal. Those (group 8) which received the basal diet plus vitamin B_{12} but without lysine grew slowly. Methionine and tryptophan (group 12), in addition to lysine, did not increase the rate of growth. In a few tests which were carried out to determine if smaller quantities of lysine would supplement cottonseed meal, 0.5% DL-lysine and 0.2% of L-lysine gave as rapid a rate of growth as 0.8% of DL-lysine. The combined average weights of all chicks which received a supplement of at least 0.5% of DL-lysine or 0.2% of L-lysine whether alone or in various combinations with methionine or tryptophan, are given in section B of table 2 (group 11). Chicks which received 0.2% DL-lysine grew only slightly faster than those which received the control diet. The amount of lysine

required to supplement different lots of cottonseed meal may vary greatly, depending upon the method by which it was prepared, but these data show that one may expect a normal rate of growth when most commercial cottonseed meals are supplemented with at least 0.2% of L-lysine or its equivalent in DL-lysine.

The chicks (group 9) which received the basal diet plus lysine without vitamin B_{12} grew much slower than those (group 10) which received the vitamin. It is obvious that the cottonseed meal was deficient in vitamin B_{12}.

Soybean oil meal as a supplement to cottonseed meal

It seemed possible, with vitamin B_{12} available to alleviate the deficiency of this vitamin in plant proteins, that one plant protein concentrate could be used to supplement the amino acid deficiency of another plant protein. In order to test this possibility, chicks were given a diet which contained cerelose, 20% of soybean oil meal and 30% of cottonseed meal, with and without vitamin B_{12}. The soybean oil meal supplied 8.4% of the total protein and the cottonseed meal supplied 11.6%. These data are summarized in section C, table 2. The chicks (group 15) which received vitamin B_{12} grew as fast as when all the protein was furnished by soybean oil meal (group 6). Without vitamin B_{12} the chicks (group 14) grew at a slower rate. Another diet which contained milo, 15% of soybean oil meal and 25% of cottonseed meal supported a rapid rate of growth when supplemented with vitamin B_{12}. These data show that it is possible to utilize plant proteins of low nutritive value by supplementing them with another plant protein of high nutritive value, if the diet is also supplemented with vitamin B_{12}.

SUMMARY

Soybean oil meal and a commercial cottonseed meal low in gossypol were investigated as the sole sources of protein for growing chicks. Soybean oil meal supported a normal rate

of growth when it was supplemented with a vitamin B_{12} concentrate equivalent to 10 to 20 μg of crystalline vitamin B_{12} per kilogram of diet. Cottonseed meal was equal to soybean oil meal when it was supplemented with vitamin B_{12} and 0.2% of L-lysine. Cottonseed meal with lysine but without vitamin B_{12} gave a subnormal rate of growth, which showed that it was deficient in this vitamin.

Soybean oil meal was used to supplement the lysine deficiency of cottonseed meal. The combination of the two protein concentrates supported as rapid a rate of growth when supplemented with vitamin B_{12} as did soybean oil meal alone. Without vitamin B_{12} growth was at a subnormal rate. These data suggest that plant proteins of relatively low value may be used as a source of protein for growing chicks when they are supplemented with soybean oil meal and vitamin B_{12}.

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